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TO: Assistant Commissioner for Patents
Box Patent Applications
Washington D.C. 20231

Attorney Docket No. 79997/124

(must include alphanumeric codes if no inventors named)

UTILITY PATENT APPLICATION TRANSMITTAL
(new nonprovisional applications under 37 CFR 1.53(b))

Transmitted herewith for filing is the patent application of:

INVENTOR(S): Cherk Shing TAM

TITLE: BONE STIMULATING FACTOR

In connection with this application, the following are enclosed:

APPLICATION ELEMENTS:

☒ Specification - 79 TOTAL PAGES

(preferred arrangement:)

- Descriptive Title of the Invention
- Cross Reference to Related Applications
- Statement Regard Fed sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

☒ Drawings - Total Sheets 13

Declaration and Power of Attorney - Total Sheets

 Newly executed (original or copy)

 Copy from a prior application (37 CFR 1.63(d))

(relates to continuation/divisional boxes completed) - NOTE: Box below

 DELETION OF INVENTOR(S) - Signed statement attached deleting inventor(s)
named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

 Incorporation By Reference (useable if copy of prior application
Declaration being submitted)

The entire disclosure of the prior application, from which a COPY of the
oath or declaration is supplied as noted above, is considered as being
part of the disclosure of the accompanying application and is hereby
incorporated by reference therein.

 Microfiche Computer Program (Appendix)

 Nucleotide and/or Amino Acid Sequence Submission (if applicable,
all necessary)

 Computer Readable Copy

 Paper Copy (identical to computer copy)

 Statement verifying identify of above copies

ACCOMPANYING APPLICATION PARTS

 Assignment Papers (cover sheet & document(s))

 37 CFR 3.73(b) Statement (when there is an assignee)

 English Translation Document (if applicable)

 Information Disclosure Statement (IDS) with PTO-1449. Copies of IDS Citations

 Preliminary Amendment

☒ Return Receipt Postcard (MPEP 503)

☐ Small Entity Statement(s)
☐ Statement file in prior application, status still proper and desired.
☐ Certified Copy of Priority Document(s) with Claim of Priority
(if foreign priority is claimed).
☐ OTHER:

If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP)
of prior application Serial No. _____.

☐ Amend the specification by inserting before the first line the following sentence: --This application is a _____ continuation, _____ divisional or _____ continuation-in-part of application Serial No. _____, filed _____.--

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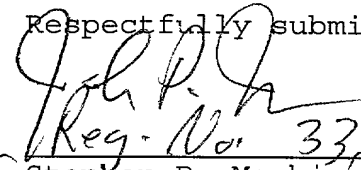
FEE CALCULATIONS: (Small entity fees indicated in parentheses.)

(1) For	(2) Number Filed	(3) Number Extra	(4) Rate	(5) Basic Fee \$760 (\$380)
Total Claims	49 - 20 =	29	x \$18 (x \$9)	\$522.00
Independent Claims	7 - 3 =	4	x \$78 (x \$39)	\$312.00
Multiple Dependent Claims			\$260 (\$130)	0.00
Surcharge Under 37 C.F.R. 1.16(e)			\$130 (\$65)	\$130.00
			TOTAL FEE:	\$1,724.00

METHOD OF PAYMENT:

If payment by check is NOT enclosed, it is requested that the Patent and Trademark Office advise the undersigned of the period of time within which to file the TOTAL FEE.

Date: June 2, 1999
Docket No.: 79997/124

Respectfully submitted,

for Reg. No. 33,715
Stephen B. Maebius
Reg. No. 35,264

BONE STIMULATING FACTOR

This application is a continuation-in-part application of international patent application No. PCT/CA 97/00967 filed December 11, 1997, designating the United States, United States Patent Application Serial No. 08/763,458, filed December 11, 1996,
5 international patent application No. PCT/CA 96/00401 filed June 7, 1996, all of which applications are incorporated herein by reference.

The present invention relates to polypeptides which stimulate bone growth.

Understanding of issues related to bone growth and strength has progressed over the years, a summary being provided in international patent application No.
10 PCT/CA 94/00144, published under international publication No. WO 94/20615 on September 15, 1994.

Various approaches to treatment of diseases involving reduction of bone mass and accompanying disorders are exemplified in the patent literature. For example, United States Patent No. 4,877,864, issued October 31, 1989 describes human and bovine
15 "bone inductive factors." International patent application published September 17, 1992 under No. 92/15615 describes a protein derived from a porcine pancreas which acts to depress serum calcium levels for treatment of bone disorders that cause elevation of serum calcium levels. European Patent Application No. 504 938 published September 23, 1992 describes the use of di- or tripeptides which inhibit cysteine protease in the treatment of bone
20 diseases. International patent application published September 3, 1992 under No. 92/14481 discloses a composition for inducing bone growth, the composition containing activin and bone morphogenic protein. European Patent Application No. 499 242 published August 19, 1992 describes the use of cell growth factor compositions thought to be useful in bone diseases involving bone mass reduction because they cause osteoblast proliferation.
25 International patent application published June 25, 1992 under No. 92/10515 1992 describes a drug containing the human N-terminal parathyroid hormone (PTH) fragment 1 - 37. European Patent Application No. 451 867 published September 16, 1991 describes parathyroid hormone peptide antagonists for treating dysbolism associated with calcium or phosphoric acid, such as osteoporosis. United States Patent No. 5,461,034 issued October
30 24, 1995 to Yissum Research Development Company of the Hebrew University of Jerusalem describes osteogenic growth polypeptides identified from regenerating bone marrow.

A relatively short half life of PTH in the blood serum and the positive effect of intermittent PTH injection on bone volume led the present investigator to the hypothesis that PTH may in some way lead to induction of a second factor into the circulatory system. The
35 presence of such a second factor in blood serum of rats and of humans has thus been investigated.

It has been found possible to isolate from rat blood serum a polypeptide substance which, upon administration to rats incapable of producing PTH (parathyroidectomized rats), produces an increase in the observed bone mineral apposition

rate. A nucleic acid probe, based on the amino acid sequence of the rat peptide was synthesized and used to screen a human liver cDNA fetal library in order to isolate a human nucleic acid sequence coding for a human bone apposition polypeptide. A polypeptide derived from the nucleic acid sequence was thus chemically synthesized according to the

5 derived sequence Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn Gln Pro (SEQ ID NO:1). It has been observed that the bone apposition rate in intact rats increases in a dose dependent fashion upon administration of this chemically synthesized compound. Reduced bone growth, normally observed for ovariectomized rats, was observed not to occur in rats after being

10 administered with the polypeptide over a four week period beginning two weeks after ovariectomization. Bone calcium density was found to be maintained in ovariectomized rats administered with the polypeptide over an eight week period beginning eight weeks after ovariectomization.

It is thought possible that the active polypeptide is a dimer of the foregoing

15 sequence, there being evidence of significant dimer formation, presumably due to a disulfide bridge between two polypeptides having the sequence shown.

A modified form of the polypeptide containing a cys→ala substitution was thus synthesized: Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Ala Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn Gln Pro (SEQ ID NO:3).

20 Some of the bone stimulatory effects of the "normal" polypeptide (SEQ ID NO:1) were found for the modified polypeptide.

In other experiments, the bone mineral apposition rate in rats administered with rabbit antibodies to the normal polypeptide (SEQ ID NO:1) was found to be suppressed. The suppression was found to be attenuated in rats administered with both the normal

25 polypeptide and antibodies to same.

Further, certain polypeptide fragments of the normal polypeptide (SEQ ID NO:1) have been synthesized and each has been found to have bone stimulatory effects:

SEQ ID NO:4:
Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys

30 Ala Ala Glu Thr Leu Met Val
SEQ ID NO:5:
Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala
SEQ ID NO:6:
Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu

35 SEQ ID NO:7:
Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile
SEQ ID NO:8:
Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys

SEQ ID NO:9:

Arg Thr Asn Glu His Thr Ala Asp Cys Lys

Further, the polypeptide identified as SEQ ID NO: 7 has been found to increase bone calcium content of ovariectomized rats when administered over a period of
5 eight or twelve weeks.

Other polypeptide fragments of the normal polypeptide (SEQ ID NO: 1) have also been synthesized and have been found to lack the bone stimulatory effect found for the normal polypeptide:

SEQ ID NO:10:

10 Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn Gln

SEQ ID NO:11:

Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn

SEQ ID NO:12:

Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln

15 SEQ ID NO:13:

Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp

SEQ ID NO:14:

Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu
Asp

20 SEQ ID NO:15:

Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr
Leu Met Val Leu Asp Gln Asn

SEQ ID NO:16:

Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile

25

The polypeptide identified as SEQ ID NO:9 was modified to include a protecting group at each end. The N-terminus was thus acetylated and C-terminus was amidated. The activity of this protected polypeptide, identified as SEQ ID NO:24, was found to increase the bone mineral apposition rate in rats beyond that observed for each of the polypeptides identified as SEQ ID NOs: 1,7 and 9.

30

It has been reported that histidine and cysteine residues can effect degradation of asparaginyl- and aspartyl-containing polypeptides in the absence of catalytic enzymes [Int. J. Peptide Protein Res. 45, 1995, 547,553]. The following analogues of the polypeptide identified as SEQ ID NO:9 were tested, for stability and for effects on bone mineral apposition rate:

35

SEQ ID NO:25

CH₃CO - Arg Thr Asn Glu His Thr Ala Glu Cys Lys - NH₂

SEQ ID NO:26

CH₃CO - Arg Thr Gln Glu His Thr Ala Glu Cys Lys - NH₂

SEQ ID NO:27

CH₃CO - Arg Thr Gln Glu His Thr Ala Asp Cys Lys - NH₂

In terms of stability under the various conditions tested, the polypeptides identified as SEQ ID NOS:25 and 26 were found to be more stable than those identified as SEQ ID NOS:9, 7 and 24. The polypeptide identified as SEQ ID NO:27 was found to be less

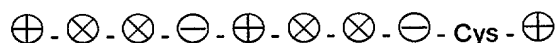
5 stable than any of SEQ ID NOS: 7, 9, 24, 25 and 26.

Each of the polypeptides identified as SEQ ID NOS:24, 25, 26 and 27 were found to increase the bone apposition rate over that observed for control rats.

Polypeptide sequences corresponding to SEQ ID NOS:25, 26 and 27 in which the terminal amino acid residues are not protected are referred to herein as SEQ ID NOS:28, 29 and 30, respectively.

10

It has further been found, by a series of substitutions that the general charge pattern, based on the side chains of the component amino acids, is important to the activity of the 10-amino sequences identified as SEQ ID NOS:9, 24, 25 26 and 27:



15 In the case of each side chain indicated by an "X", the side chain does not bear a full ionic charge under physiological conditions. An equivalent way of describing this arrangement of charges within the polypeptide is to say that the polypeptide has an amino acid sequence of up to 10 + q amino acids, wherein, under physiological conditions, residues numbered n, n + 4, n + 9 are positively charged amino acids, residues numbered n + 3, n + 7 are negatively

20 charged amino acids, wherein the remaining amino acids are nonpolar amino acids or uncharged polar amino acids, and wherein n is an integer from 1 to 1 + q. As polypeptides having between 10 and 36 amino acids have been shown to have bone stimulatory activity, q can be from 0 up to 26. In particular embodiments, q is up to 20, 15, 14, 10, and 5.

Particular sequences shown to retain bone stimulatory activity include those in which substitutions have been made as follows:

25 R - T/A - N/Q/A - E - H - T/A - G/A - E/D - C/Y/A/S - K

As can be seen, each of the threonine residues in the second and sixth positions, respectively, of the sequence can be substituted by alanine. The oxygen atom contained in the side chain of threonine is generally considered to impart a negative polar

30 charge to the side chain while the hydrocarbon side chain of alanine is generally considered to be non-polar. At the third position of the sequence, the asparagine residue has a side chain that contains the polar amide group and this can be interchanged with glutamine, having a similarly polar amide group in its side chain, or it can be interchanged with alanine having a non-polar side chain. In the case of the seventh position of the sequence, a glycine

35 which has only a hydrogen radical as a side chain and which would not be considered to be polar, can be interchanged with the alanine residue. In the eighth position of the illustrated sequence, the aspartic acid residue can be replaced by a glutamic acid residue, both of which have side chains that include carboxylic acid groups, which under physiological conditions

can be deprotonated and thus have a negative charge. In the ninth position, there is normally a cysteine residue which contains an "SH" group and this has been shown to react with the SH group of a second molecule resulting in a dimer. The cysteine residue can be substituted for by a tyrosine, alanine or serine with retention of bone-stimulatory activity of the compound.

5 Polypeptides in which alanine has been substituted in place of each of the amino acids having a charged side chain, SEQ NOs:34, 35, 36, 37 and 38, were found to lack or to exhibit substantially less bone stimulatory activity than the family of compounds in which the full general charge pattern and spacing was retained. On the other hand, sequences in which the second, third, sixth and seventh amino acids were substituted by alanine (or glycine
10 in the case of the seventh amino acid, which is alanine in the parent sequence, SEQ ID NO:9), SEQ ID NOs: 39, 40, 41 and 42, largely retain bone stimulatory activity.

A polypeptide in which the ninth amino acid, cysteine, has been replaced by tyrosine (SEQ ID NO:43) was found to have some bone stimulatory activity.

15 A polypeptide in which the third amino acid, asparagine, has been replaced by glutamine, the eighth amino acid, aspartic acid, has been replaced by glutamic acid, and the ninth amino acid, cysteine, has been replaced by alanine (SEQ ID NO:44) was found to have bone stimulatory activity.

20 A polypeptide in which the third amino acid, asparagine, has been replaced by glutamine, the eighth amino acid, aspartic acid, has been replaced by glutamic acid, and the ninth amino acid, cysteine, has been replaced by tyrosine (SEQ ID NO:45) was found to have bone stimulatory activity.

25 A polypeptide in which the third amino acid, asparagine, has been replaced by glutamine, the eighth amino acid, aspartic acid, has been replaced by glutamic acid, and the ninth amino acid, cysteine, has been replaced by serine (SEQ ID NO:46) was found to have bone stimulatory activity.

30 The present invention thus includes a compound having bone stimulatory activity in mammals, the compound being derived from a polypeptide having the charge pattern of the amino acid side chain charges provided by the amino acid sequence corresponding to SEQ ID NO:9. The backbone of the compound is preferably substantially isosteric with that provided by the peptide backbone of the amino acid sequence corresponding to SEQ ID NO:9. In a particular aspect, the compound is itself a polypeptide.

35 In a particular aspect, for example, polypeptides identified as SEQ ID NOs:24, 25, 26, 27, 39, 40, 41, 42, 43, 44, 45 and 46 the charge pattern of the compound consists essentially of that provided by the amino acid sequence corresponding to SEQ ID NO:9, that is, it bears side chain charges in the order of and spaced as the amino acid side chains of SEQ ID NO:9 and does not include other amino acids. The invention includes a compound with substitutions of the sequence corresponding to SEQ ID NO:9 which retain bone stimulatory activity in mammals.

In one aspect, the invention is a compound that is a polypeptide and the sequence from which the polypeptide is derived consists of up to 25 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1 and includes the charge pattern provided by SEQ ID NO:9. Such a compound thus has a polypeptide sequence that "consists of up to 25 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1". The compound can have more than 25 amino acids, but no single portion (string of contiguous amino acid residues) of the entire sequence has more than 25 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1.

In another aspect, the invention includes a compound having bone stimulatory activity in mammals, in which the compound has the charge distribution of the side chain charges provided by the amino acid sequence corresponding to SEQ ID NO:9 and having up to about 83% sequence homology with the parent sequence identified as SEQ ID NO:1. The invention also includes a polypeptide having bone stimulatory activity in mammals in which the polypeptide has the charge distribution of the side chain charges provided by the amino acid sequence identified as SEQ ID NO:9 and including at least one non-conservative substitution at position number 2, 3, 6 or 9 of SEQ ID NO:9.

The present invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:1 with (a) from one to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:1 (b) one to about 22 amino acids deleted from the C-terminus of SEQ ID NO:1, or both (a) and (b); or a functionally equivalent homologue. Correspondingly, the invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:3 with (a) from one to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:3 (b) one to about 22 amino acids deleted from the C-terminus of SEQ ID NO:3, or both (a) and (b); or a functionally equivalent homologue. Sequence homology in polypeptides and proteins is understood to those skilled in the art, as discussed, for example in Molecular Cell Biology (H. Lodish, D. Baltimore, A. Berk, S.L. Zipursky, P. Matsudaira and J. Darnell, Scientific American Books, New York City, Third Edition, 1995). Likewise, the invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:4 with (a) up to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:4, (b) up to about 16 amino acids deleted from the C-terminus of SEQ ID NO:4, or both (a) and (b); or a functionally equivalent homologue. The invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:5 with (a) up to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:5, (b) up to about 11 amino acids deleted from the C-terminus of SEQ ID NO:5, or both (a) and (b); or a functionally equivalent homologue. The invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:6 with (a) up to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:6, (b) up to about 5 amino acids deleted from the C-terminus of SEQ ID NO:6, or both (a) and (b); or a functionally equivalent homologue. The invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:7 with (a) up to about four 4 amino acids

deleted from the N-terminus of SEQ ID NO:7, (b) up to about 1 amino acids deleted from the C-terminus of SEQ ID NO:4, or both (a) and (b); or a functionally equivalent homologue. The invention also includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:8 with up to about four 4 amino acids deleted from the N-terminus or a functionally
5 equivalent homologue. The invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:9 or a functionally equivalent homologue thereof.

The invention includes a polypeptide up to about 30 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:9 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide
10 can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

The invention includes a polypeptide of up to about 25 amino acids in length
15 comprising an amino acid sequence corresponding to SEQ ID NO:9 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen
20 thereof is bound to two hydrogen atoms.

Alternatively, the invention includes a polypeptide of up to about 20 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:9 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl
25 group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms. The C-terminal and/or the N-terminal of any polypeptide of the invention can be protected, by conventional or other means.

The invention includes a polypeptide of up to about 15 amino acids in length
30 comprising an amino acid sequence corresponding to SEQ ID NO:9 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen
35 thereof is bound to two hydrogen atoms.

The invention includes a polypeptide about 10 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:9 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both.

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The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms. The invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:24.

5 The invention includes a polypeptide up to about 30 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:28 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by
10 conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

 The invention includes a polypeptide of up to about 25 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:28 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide
15 can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

 Alternatively, the invention includes a polypeptide of up to about 20 amino
20 acids in length comprising an amino acid sequence corresponding to SEQ ID NO:28 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example,
25 the amino nitrogen thereof is bound to two hydrogen atoms.

 The invention includes a polypeptide of up to about 15 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:28 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both.
30 The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

 The invention includes a polypeptide about 10 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:28 or a functionally
35 equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen

thereof is bound to two hydrogen atoms. The invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:25.

The invention includes a polypeptide up to about 30 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:29 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

The invention includes a polypeptide of up to about 25 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:29 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

Alternatively, the invention includes a polypeptide of up to about 20 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:29 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

The invention includes a polypeptide of up to about 15 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:29 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

The invention includes a polypeptide about 10 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:29 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms. The invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:26.

The invention includes a polypeptide up to about 30 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:30 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both.

- 5 The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

The invention includes a polypeptide of up to about 25 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:30 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

- 10
15 Alternatively, the invention includes a polypeptide of up to about 20 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:30 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

- 20
25 The invention includes a polypeptide of up to about 15 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:30 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms.

- 30 The invention includes a polypeptide about 10 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:30 or a functionally equivalent homologue thereof which promotes bone growth in mammals. The polypeptide can have a protected terminal amino group, or a protected terminal carboxyl group, or both. The N-terminal protecting group can be an acetyl group. The C-terminal can be protected by conversion of the carboxyl group to an amide group, in which for example, the amino nitrogen thereof is bound to two hydrogen atoms. The invention includes a polypeptide having an amino acid sequence corresponding to SEQ ID NO:27.

Polypeptides of the present invention can be incorporated into larger polypeptide sequences in which there is repetition of active sequences in a single molecule.

The inventive polypeptide can be synthetic and the amino acid sequence can have a molecular weight in the range of from about 1000 to 4000.

- The invention includes a polypeptide having a sequence of amino acids sufficiently duplicative of another, i.e., second polypeptide having an amino acid sequence
- 5 corresponding to SEQ ID NO:1 (or SEQ ID NO:3) with (a) from one to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:1 (or SEQ ID NO:3), (b) one to about 22 amino acids deleted from the C-terminus of SEQ ID NO:1 or (SEQ ID NO:3), or both (a) and (b), or a functionally equivalent homologue thereof, such that the polypeptide is encoded by a DNA that hybridizes under stringent conditions with DNA encoding the second polypeptide.
- 10 The polypeptide can be up to about 30 amino acids in length, for example, and the sequence of that polypeptide can be repeated within a larger polypeptide, or contain other polypeptide sequences which are not by themselves stimulate bone growth. Such polypeptide could also be up to 25, 20, 15 or about 10 amino acids in length.

- "Sequence identity or homology", as used herein, refers to the sequence
- 15 similarity between two polypeptide molecules or between two nucleic acid molecules. When a position in both of the two compared polypeptide sequences, for example, is occupied by the same amino acid (for example, if a position in each of two polypeptide molecules is an alanine residue, then the molecules are homologous or sequences are identical at that position. The percent of homology between two molecules or sequence identity between two
- 20 sequences is a function of the number of such matching positions shared by the two sequences divided by the number of positions compared x 100. For example, if 6 of 10, of the positions in two sequences are the same then the two sequences are 60% homologous or have 60% sequence identity. By way of example, the polypeptide sequences METLIA and MPTWIF share 50% homology or sequence identity. Generally, a comparison is made when
- 25 two sequences are aligned to give maximum homology.

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. The alignment can be performed according to two methods, the Clustal method and the J. Hein method.

- 30 The Clustal algorithm (using software available from DNASTAR Inc., 1228 South Park Street, Madison, Wisconsin, USA, 1994) is recommended for aligning sequences whose similarity might not necessarily be evolutionary. The algorithm is described by Higgins, D.G. et al. 1989. CABIOS 5:151. The same software programme provides for aligning sequences according to the Jotun Hein method, which is recommended for aligning
- 35 sequences of highly evolved families that have clear evolutionary relationship. The algorithm is described by Hein, J. 1990. Methods in Enzymology 183:626. Programme default settings (standard parameters) can be used. In the case of weighting amino acid residues based on evolutionary substitution patterns, charge, structural and chemical similarity, the default

PAM250 setting can be used. For protein alignments, the pairwise alignment parameters are Ktuple =1, Gap penalty =3, Window =5, and Diagonals Saved =5 can be used.

The phrase "selectively hybridizing to" refers to a nucleic acid probe that, under appropriate hybridization conditions, hybridizes, duplexes or binds only to a particular target DNA or RNA sequence when the target sequences are present in a preparation of DNA or RNA. "Complementary" or "target" nucleic acid sequences refer to those nucleic acids that selectively hybridize to a nucleic acid probe. Proper annealing conditions depend, for example, upon a probe's length, base composition, and the number of mismatches and their position on the probe, and must often be determined empirically. For discussions of nucleic acid probe design and annealing conditions, see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or *Current Protocols in Molecular Biology*, F. Ausubel et al., (ed.) Greene Publishing and Wiley-Interscience, New York (1987).

"Stringent hybridization conditions" takes on its common meaning to a person skilled in the art here. Appropriate stringency conditions which promote nucleic acid hybridization, for example, 6x sodium chloride/sodium citrate (SSC) at about 45°C are known to those skilled in the art. The following examples are found in *Current Protocols in Molecular Biology*, John Wiley & Sons, NY (1989), 6.3.1-6.3.6: For 50 ml of a first suitable hybridization solution, mix together 24 ml formamide, 12 ml 20x SSC, 0.5 ml 2 M Tris-HCl pH 7.6, 0.5 ml 100x Denhardt's solution, 2.5 ml deionized H₂O, 10 ml 50% dextran sulfate, and 0.5 ml 10% SDS. A second suitable hybridization solution can be 1% crystalline BSA (fraction V), 1 mM EDTA, 0.5 M Na₂HPO₄ pH 7.2, 7% SDS. The salt concentration in the wash step can be selected from a low stringency of about 2x SSC at 50°C to a high stringency of about 0.2x SSC at 50°C. Both of these wash solutions may contain 0.1% SDS. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C to high stringency conditions, at about 65°C. The cited reference gives more detail, but appropriate wash stringency depends on degree of homology and length of probe. If homology is 100%, a high temperature (65°C to 75°C) may be used. If homology is low, lower wash temperatures must be used. However, if the probe is very short (<100bp), lower temperatures must be used even with 100% homology. In general, one starts washing at low temperatures (37°C to 40°C), and raises the temperature by 3-5°C intervals until background is low enough not to be a major factor in autoradiography.

In another aspect the invention is a synthetic polypeptide having in vivo bone stimulatory activity in mammals and which increases mineral content (i.e., calcium) in bones of mammals, having an amino acid sequence which is at least about 19% conserved in relation to the amino acid sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom, or a functionally equivalent homologue.

The invention includes a synthetic polypeptide having in vivo bone stimulatory activity in mammals and which increases mineral content in bones of mammals, having an

amino acid sequence which is at least about 22% conserved in relation to the amino acid sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom.

The invention includes a synthetic polypeptide having in vivo bone stimulatory activity in mammals and which increases mineral content in bones of mammals, having an

5 amino acid sequence which is at least about 25% conserved in relation to the amino acid sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom.

The invention includes a synthetic polypeptide having in vivo bone stimulatory activity in mammals and which increases mineral content in bones of mammals, having an

amino acid sequence which is at least about 28% conserved in relation to the amino acid

10 sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom.

The invention includes any of the foregoing synthetic polypeptides in which at least six amino acids deleted from the polypeptide sequence; or in which at least eleven

amino acids deleted from the sequence; or in which at least sixteen amino acids deleted from

the sequence; or in which at least twenty-one amino acids deleted from the sequence; or in

15 which at least twenty-six amino acids deleted from the sequence.

The invention includes a polypeptide having a sequence of amino acids sufficiently duplicative of one of the foregoing synthetic polypeptides such that the polypeptide is encoded by a DNA that hybridizes under stringent conditions with DNA encoding the synthetic polypeptide.

20 In another aspect the invention is a polypeptide exhibiting bone stimulatory activity in mammals, the polypeptide having the sequence identified as SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9; SEQ ID NO:24; SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:29; or SEQ ID NO:30; analogues thereof wherein the amino acids in the

25 sequence may be substituted, deleted or added, so long as the bone stimulatory activity in mammals derived the three dimensional structure of the sequence is preserved; and

conjugates of each of the polypeptides or analogues thereof, wherein if the polypeptide sequence has that identified as SEQ ID NO:1, then there is at least one amino acid deleted therefrom. The invention includes a polypeptide that has a sequence of amino acids

sufficiently duplicative of such a bone stimulatory polypeptide (or a functionally equivalent

30 homologue thereof) that the polypeptide is encoded by a DNA that hybridizes under stringent conditions with DNA encoding the bone stimulatory polypeptide.

In another aspect, the invention is a polypeptide that includes an amino acid sequence that is between 19% and 90% conserved in relation to the amino acid sequence

35 identified as SEQ ID NO:1; or an amino acid sequence that is between 19% and 86%

conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino

acid sequence that is between 19% and 69% conserved in relation to the amino acid

sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 19% and

56% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an

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amino acid sequence that is between 19% and 42% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 19% and 39% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 19% and 28% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 28% and 90% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 28% and 86% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 28% and 69% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 28% and 56% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 28% and 42% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or an amino acid sequence that is between 28% and 39% conserved in relation to the amino acid sequence identified as SEQ ID NO:1; or a functionally equivalent homologue that has bone stimulatory activity in a mammal.

In another aspect, the invention is a polypeptide having bone stimulatory activity, the polypeptide comprising an amino acid sequence that has $10 + q$ amino acids, wherein, under physiological conditions, residues numbered n , $n + 4$, $n + 9$ are positively charged amino acids, residues numbered $n + 3$, $n + 7$ are negatively charged amino acids, wherein the remaining amino acids are nonpolar amino acids or uncharged polar amino acids, wherein n is an integer from 1 to $1 + q$ and q is an integer from 0 to 15; and, wherein the polypeptide includes no more than 25 consecutive amino acids corresponding to the amino acid sequence identified as SEQ ID NO:1.

A person skilled in the art would of course, understand that modifications of sequences, such as those identified as SEQ ID NO:1 or SEQ ID NO:9, described herein are to be each taken as though made separately and independently of each other.

In the case of this aspect of the invention, q can be 10, 5 or 0. The remaining amino acids can be selected from the group consisting of glycine, alanine, valine, isoleucine, serine, threonine, methionine, asparagine and glutamine. The residue numbered $n + 8$ can be cysteine, tyrosine, alanine or serine; the residue numbered n can be arginine; the residue numbered $n + 1$ can be alanine or threonine; the residue numbered $n + 2$ can be alanine, asparagine, or glutamine; the residue numbered $n + 3$ can be glutamic acid; the residue numbered $n + 4$ can be histidine; the residue numbered $n + 5$ can be threonine or alanine; the residue numbered $n + 6$ can be glycine or alanine; the residue numbered $n + 7$ can be glutamic acid or aspartic acid; and the residue numbered $n + 9$ can be lysine.

The polypeptide can be a chimeric bone stimulating factor that includes any of the amino acid sequences described above as part of the invention.

The invention includes an agent for use in prevention and treatment of a bone reduction related disease that includes any polypeptide described above as part of the invention, including of course a chimeric polypeptide, as an active ingredient.

5 The invention is thus also a pharmaceutical composition for promoting bone growth, having a therapeutically effective amount of any polypeptide described above as part of the invention.

The invention includes a method of increasing bone growth in a mammal by administering a therapeutically effective amount of a polypeptide (or a pharmaceutical composition including the polypeptide) described above as part of the invention.

10 The invention includes the treatment of osteoporosis, promotion of bone growth in a mammal or treatment of a human of a bone reduction related disease.

The invention includes the use of a polypeptide having a sequence according to any polypeptide of the invention in the preparation of a medicament for use in promoting bone growth or the treatment of osteoporosis, etc.

15 The invention includes a diagnostic kit for determining the presence of a polypeptide of the invention, in which the kit includes an antibody to a polypeptide (or polypeptides) linked to a reporter system wherein the reporter system produces a detectable response when a predetermined amount of the polypeptide (or polypeptides) and the antibody become bound together.

20 The invention includes an antibody which binds to a polypeptide of the invention. Particularly, the invention includes an antibody which binds to such a polypeptide when the antibody is synthesized using the polypeptide.

The invention includes molecules, such as isolated nucleotide sequences related to polypeptides of the invention. For example, the invention includes an isolated DNA
25 fragment which encodes the expression of any of the polypeptides of the invention. It is of course understood that such fragments can vary from one another due to the degeneracy of the genetic code. Further, the invention includes a vector that has incorporated into it any such DNA sequence.

30 The invention includes an isolated DNA sequence encoding any amino acid sequence of the invention, or an analogue thereof, wherein the amino acids in the sequence may be substituted, deleted or added, so long as bone stimulatory activity in mammals derived from the three dimensional conformation of the sequence is preserved in a polypeptide having the amino acid sequence; sequences which hybridize to the DNA and encode an amino acid sequence of a polypeptide which displays bone stimulatory activity in
35 mammals; and DNA which differs from the sequence due to the degeneracy of the genetic code.

The invention thus includes processes of producing any polypeptide of the invention, including a process which includes: a) preparing a DNA fragment containing a nucleotide sequence that encodes such a polypeptide; b) incorporating the DNA fragment into

- an expression vector to obtain a recombinant DNA fragment which contains the DNA fragment and is capable of undergoing replication; c) transforming a host cell with the recombinant DNA fragment to isolate a transformant which can express the polypeptide; and d) culturing the transformant to allow the transformant to produce the polypeptide and recovering the polypeptide from resulting cultured mixture.

BRIEF DESCRIPTION OF THE DRAWINGS

In the following description, reference is made to accompanying drawings, wherein,

- Figure 1 graphically depicts the bone mineral apposition rate (μm per day) in rats provided with the chemically synthesized human N-acetyl (N-terminus) polypeptide (SEQ ID NO:2) through implantation in parathyroidectomized rats. The error bars indicate ± 1 standard deviation (S.D.). The value of p was less than 0.001.

- Figure 2 graphically depicts right femoral bone calcium density of rats treated over a four week period. Group A rats were ovariectomized and injected daily with the chemically synthesized normal peptide (SEQ ID NO:1). Group B rats were ovariectomized and injected daily with control solution. Group C rats were subject to sham ovariectomization operations and injected daily with control solution. Group D were intact rats injected daily with control solution. The error bars indicate ± 1 standard deviation (S.D.).

- Figure 3 graphically depicts the bone mineral apposition rate of rats as determined by tetracycline labelling after treatment as described in connection with Figure 2. The error bars indicate ± 1 standard deviation (S.D.).

- Figure 4 graphically depicts femoral bone calcium concentration of rats treated over an eight week period. Group A rats were ovariectomized and injected daily with the chemically synthesized normal peptide (SEQ ID NO:1) beginning eight weeks after the operation. Group B rats were similarly ovariectomized and injected daily with control solution. Group C rats were subject to sham ovariectomization operations and injected daily with control solution. Group D were intact rats injected daily with control solution. The error bars indicate ± 1 standard deviation (S.D.).

- Figure 5 graphically depicts the bone mineral apposition rate of intact rats as determined by tetracycline labelling. Group A rats were treated with rabbit antibodies to the chemically synthesized normal polypeptide (SEQ ID NO:1). Group B rats were treated with the same antibodies and the polypeptide itself. Group C is the control group. The error bars indicate ± 1 standard deviation (S.D.).

- Figure 6 shows a tricine SDS electrophoretic gel of the human chemically synthesized polypeptide (SEQ ID NO:1) and the same polypeptide containing a cys \rightarrow ala substitution (SEQ ID NO:3).

Figure 7 graphically depicts the bone mineral apposition rate (μm per day) in rats injected with the chemically synthesized human polypeptide (SEQ ID NO:1), Group A; the

modified chemically synthesized human polypeptide (SEQ ID NO:3), Group B; and control, Group C. (N=6 for all groups). The error bars indicate ± 1 standard deviation (S.D.).

Figure 8 graphically depicts the bone mineral apposition rate (μm per day) in rats injected with N-terminus chemically synthesized polypeptides: SEQ ID NO:1 (Group A);
5 SEQ ID NO:7 (Group B); SEQ ID NO:6 (Group C); SEQ ID NO:5 (Group D); and SEQ ID NO:4 (Group E). (N=6 for all groups). The error bars indicate ± 1 standard deviation (S.D.).

Figure 9 graphically depicts the bone mineral apposition rate (μm per day) in rats injection with chemically synthesized polypeptides: SEQ ID NO:8 (Group F); SEQ ID NO:9 (Group G).

10 Figure 10 is a DEXA image of a right femur of a rat showing scanned areas: A, proximal end; B, diaphysis; and C, distal end .

Figure 11 is a DEXA image of a right femur of a rat showing scanned neck area.

Figure 12 graphically depicts the bone mineral apposition rate (μm per day) in
15 rats injected with non-N-terminus chemically synthesized polypeptide fragments SEQ ID NO:1 (Group H); SEQ ID NO:16 (Group I); SEQ ID NO:15 (Group J); SEQ ID NO:14 (Group K); and SEQ ID NOs:10,11,12 & 13 (Group L). (N=6 for all groups). The error bars indicate ± 1 standard deviation (S.D.).

Figure 13 graphically depicts the bone mineral apposition rate (μm per day) in
20 rats injected with chemically synthesized polypeptide fragments SEQ ID NO:1 (Group N); SEQ ID NO:7 (Group O); SEQ ID NO:9 (Group P); and SEQ ID NO:24 (Group Q), and a control group (Group M). The error bars indicate ± 1 standard deviation (S.D.).

Figure 14 graphically depicts the bone mineral apposition rate (μm per day) in
25 rats injected with chemically synthesized polypeptide fragments SEQ ID NO:25 (Group S); SEQ ID NO:26 (Group T); SEQ ID NO:27 (Group U); SEQ ID NO:24 (Group V) and a control group (Group R). The error bars indicate ± 1 standard deviation (S.D.).

Figure 15 graphically depicts the bone mineral apposition rate (μm per day) in
rats injected with chemically synthesized polypeptide fragments SEQ ID NO:26 (Group LL); SEQ ID NO:44 (Group MM); SEQ ID NO:45 (Group NN); and SEQ ID NO:46 (Group PP).
30 The error bars indicate ± 1 standard deviation (S.E.). $P < 0.001$, 0.05, 0.0025 and 0.01 for Groups LL, MM, NN and PP, respectively.

Figure 16 graphically depicts the bone mineral apposition rate (μm per day) in
rats injected with chemically synthesized polypeptides SEQ ID NO:24 (Group AA); SEQ ID NO:34 (Group BB); SEQ ID NO:35 (Group CC); SEQ ID NO:36 (Group DD); SEQ ID NO:37
35 (Group EE); and SEQ ID NO:38 (Group FF). The first bar of the graph is the control group. The error bars indicate ± 1 S.E.

Figure 17 graphically depicts dosage dependence of the bone mineral apposition rate (μm per day) in rats injected with chemically synthesized polypeptides SEQ ID

NO:34 (Group BB, (■)); SEQ ID NO:35 (Group CC, (▲)); SEQ ID NO:36 (Group DD, (▼)); SEQ ID NO:37 (Group EE, (◆)); and SEQ ID NO:38 (Group FF, (hexagons)). The error bars indicate ± 1 S.E.

5 Figure 18 graphically depicts the bone mineral apposition rate (μm per day) in rats injected with chemically synthesized polypeptides SEQ ID NO:39 (Group GG); SEQ ID NO:40 (Group HH); SEQ ID NO: 41 (Group II) and SEQ ID NO:42 (Group JJ). The last bar of the graph is the control group. The error bars indicate ± 1 S.E.

10 Figure 19 graphically depicts the bone mineral apposition rate (μm per day) in rats injected with chemically synthesized polypeptide having the amino acid sequence identified as SEQ ID NO:43 (Group KK). The other bar of the graph is the control group. The error bars indicate ± 1 S.E.

Figure 20 illustrates the amino sequences of the various polypeptides tested, active polypeptides being shown above the mid-line and sequences which were not found to stimulate bone growth being below the mid-line.

15 METHODOLOGY

The applicable methodology as described in the General Methodology section of international patent application No. PCT/CA 94/00144 was followed here.

20 TOXICITY EXPERIMENTS INVOLVING N-TERMINAL ACETYL CHEMICALLY SYNTHESIZED POLYPEPTIDE (SEQ ID NO: 2)

25 A miniosmotic pump (Alzet) was loaded with about 1.5 ml of the chemically synthesized peptide having an N-terminal acetyl group (SEQ ID NO:2) in 0.1% acetic acid so as to give a calculated daily delivery of about 25 μg per day. A pump was implanted under the subcutaneous fascia of the dorsal aspect of the left side of the thorax of five rats which had been parathyroidectomized seven days earlier. Five similarly parathyroidectomized rats received similar implants containing only 0.1% acetic acid. Five intact rats were also used as controls.

30 Twenty-eight days later 0.5 ml of an aqueous solution of tetracycline hydrochloride was injected intramuscularly into the right gluteus maximus of each of the implanted rats, as described previously. Another 48 hours later, a second injection of tetracycline hydrochloride solution was injected. The rats were sacrificed another 24 hours later.

35 The bone mineral apposition rate was determined by examination of a cross-section of the lower metaphysis of the right femur of each of the ten rats which had been given implants. The results are summarized in Table One depicted graphically in Figure 1.

TABLE ONE: Comparison of the Group Arithmetic Means Among Groups		
	Test Group	Control Group
Mean	1.27 $\mu\text{m/d}$	0.67 $\mu\text{m/d}$
S.D.	0.18 $\mu\text{m/d}$	0.08 $\mu\text{m/d}$
N	5	5
	t	d.f
Test Group vs Control Group	7.14	8

Histological evaluation of selected tissues of the five rats of each of the groups indicated in Table One were carried out microscopically. No evidence of toxic lesions was found.

5 EXPERIMENTS INVOLVING OVARECTOMIZED RATS AND THE NORMAL CHEMICALLY SYNTHESIZED POLYPEPTIDE (SEQ ID NO:1), ADMINISTRATION OVER A FOUR WEEK PERIOD

Ovariectomies were performed on six female Sprague-Dawley rats, each sedated with 1 mg of sodium barbiturate I.P. Sham operations were carried out a second group of six rats. The rats were given two weeks to recover from the operations.

The six ovariectomized rats were injected subcutaneously with 100 μl of a 0.1% acetic acid solution containing 100 μg of the chemically synthesized peptide (SEQ ID NO:1) every 24 hours for 28 days. On day 25, a tetracycline hydrochloride solution was injected intramuscularly into each rat so as to give 24 mg per Kg of body weight, as described previously. On day 27, a second dose of tetracycline hydrochloride was injected and the rats were sacrificed on the 28th day.

A second group of six ovariectomized rats, was similarly treated with a 0.1% acetic acid solution containing no peptide over the same 28 day period. A third group of six rats, each of which had undergone the sham operation, was similarly treated with a 0.1% acetic acid solution containing no peptide over the same 28 day period. A fourth group of six intact rats was similarly treated with a 0.1% acetic acid solution containing no peptide over the same 28 day period.

Post-mortem blood was taken by cardiac puncture and serum frozen until analyzed. A full autopsy was performed on each rat. No ill effects were observed in the rats treated with the polypeptide.

Each of the right femurs was dissected out from its soft tissue, fixed for two days, and X-rays taken at 70 kV for 1 min., 2 min., and 3 min. The 3 minute exposures gave the most satisfactory results. The bone densities of the femurs from the second group of rats, the ovariectomized rats not treated with the peptide, showed a visibly lower bone density.

The right femur of each rat was decalcified separately. The decalcification fluid consisted of 10% formic acid (v/v) and 5% sodium citrate (w/v) at pH 3.0. Each bone was placed in 6 ml of the decalcification fluid. The fluid was replaced after 4 days, again after another 4 days, again after another 2 days, and again after another 3 days. After another 2 days, the decalcification fluid was removed and replaced by deionized water, and the sample agitated for 2 days. The water changed after two days and again after another day. After another day, all of the fluid samples for each rat were combined and the final volume of each adjusted to 50 ml with deionized water.

The volume of each right femur was determined by determining the volume of water displaced when the bone was immersed in water. The calcium concentration of each sample was determined according to standard methods and the calcium density of each bone calculated. The results are tabulated in Table Two and graphically depicted in Figure 2. As can be seen, the bone calcium concentration measured for the ovariectomized rats treated with the peptide (SEQ ID NO:1) appears to be normal, while the calcium concentration of the untreated ovariectomized rats is depressed.

TABLE TWO: Right Femoral Calcium Concentration of Ovariectomized Rats				
	Group A	Group B	Group C	Group D
Mean ($\mu\text{mol/ml}$)	7.57	6.61	7.45	7.69
N	6	6	6	6
S.D.	0.38	0.29	0.28	0.31
GROUP		t	d.f.	p
A vs B		4.90	10	< 0.001
A vs C		0.62	10	> 0.5
A vs D		0.60	10	> 0.5
B vs C		5.08	10	< 0.001
B vs D		6.20	10	< 0.001
C vs D		1.40	10	> 0.1

The bone mineral apposition rate was determined, as described previously, by measurement of the lower metaphysis of the left femur. The results are tabulated in Table Three and graphically depicted in Figure 3.

5

TABLE THREE: Bone Mineral Apposition Rates of Ovariectomized Rats				
	Group A	Group B	Group C	Group D
Mean ($\mu\text{m/day}$)	0.90	0.59	0.85	0.86
N	6	6	6	6
S.D.	0.12	0.07	0.07	0.09
GROUP		t	d.f.	p
A vs B		5.39	10	< 0.001
A vs C		0.87	10	> 0.5
A vs D		0.21	10	> 0.5
B vs C		6.29	10	< 0.001
B vs D		5.93	10	< 0.001
C vs D		0.21	10	> 0.5

EXPERIMENTS INVOLVING OVARECTOMIZED RATS AND THE NORMAL CHEMICALLY SYNTHESIZED POLYPEPTIDE, ADMINISTRATION OVER AN EIGHT WEEK PERIOD

Eight weeks after ovariectomy, five ovariectomized rats were injected subcutaneously with 100 µl of a 0.1% acetic acid solution containing 100 µg of the chemically synthesized peptide in which the N-terminal amino group was modified with an acetyl group (SEQ ID NO:2). This was done every 24 hours for eight weeks. On day 54, a tetracycline hydrochloride solution was injected intramuscularly into the right gluteus maximus of each rat so as to give 24 mg per Kg of body weight, as described previously. On day 56, a second dose of tetracycline hydrochloride was injected and the rats were sacrificed on the 57th day.

A second group of seven ovariectomized rats, was similarly treated with a 0.1% acetic acid solution containing no peptide over the same period. A third group of five rats, each of which had undergone the sham operation, was similarly treated with a 0.1% acetic acid solution containing no peptide over the same period. A fourth group of five intact rats was similarly treated with a 0.1% acetic acid solution containing no peptide over the same 8 week period. Two rats of the second group became ill during the 8 week period and were sacrificed prematurely.

Post-mortem blood was taken by cardiac puncture and serum frozen until analyzed. An autopsy was performed on each rat. No obvious pathology was observed in the rats except for surgical scars and atrophy of the uterus and vagina of ovariectomized rats.

The right femurs were decalcified and calcium density determined as before. The results are presented in Table Four and Figure 4.

TABLE FOUR: Right Femoral Calcium Concentration of Ovariectomized Rats				
	Group A	Group B	Group C	Group D
Mean (µmol/ml)	7.37	6.89	7.69	7.87
N	5	5	5	5
S.D.	0.15	0.32	0.30	0.24
GROUP	t		d.f.	p
A vs B	3.85		6	< 0.005
A vs C	1.17		6	> 0.2
A vs D	3.01		6	< 0.01
B vs C	4.03		6	< 0.005
B vs D	5.41		6	< 0.001
C vs D	1.60		6	> 0.1

SYNTHESIS OF ANTIBODIES TO CHEMICALLY SYNTHESIZED PROTEIN (SEQ ID NO: 1)

The chemically synthesized protein (SEQ ID NO:1) was coupled to KLH (keyhole limpet hemocyanin) with three different cross-linkers, as described below.

5 GLUTARALDEHYDE COUPLING

2.5 ml of a PBS solution made up of 2.7 mM KCl, 1.2 mM KH_2PO_4 , 138 mM NaCl, 8.1 mM Na_2HPO_4 , were diluted 5 mg of the peptide (SEQ ID NO:1) to obtain a final peptide concentration of 2 mg/ml. 10 mg of KLH were diluted in 5.0 ml PBS to obtain a final concentration of 2 mg/ml. To 1.25 ml of the KLH solution were added 1.25 ml of the peptide solution. Glutaraldehyde was added to a final concentration of 0.25%. The resultant solution was stirred for 1 hour at room temperature. After stirring, the solution was dialysed against 1 litre of PBS. The PBS was changed three times.

CARBODIIMIDE (EDC) COUPLING

Peptide and KLH solutions were prepared as described in the preceding section. To 1.25 ml KLH solution were added 1.25 ml peptide solution. To the resultant solution were added 2.5 mg of EDC. The solution was stirred constantly at room temperature for 4 hours and then dialysed against 1 litre of PBS. The PBS was changed three times.

M-MALEIMIDOBENZOYL-N-HYDROXSUCCINIMIDE ESTER (MBS) COUPLING

To 500 μl of H_2O were added 5 mg of the peptide and the pH was adjusted to 8.5 with NaOH, to obtain a final concentration of 10 mg/ml. Citraconic anhydride was diluted in H_2O to a concentration of 10 mg/ml. 500 μl of the anhydride solution were added to the peptide solution 100 μl at a time with adjustment of the pH to 8.5 between each addition. The solution was then stirred constantly at room temperature for 1 hour. This was followed by the addition of 100 μl of 1M sodium phosphate buffer (pH 7.2) and then 900 μl of 100 mM sodium phosphate buffer (pH 7.2). Sulfo-MBS was diluted in H_2O to a concentration of 25 mg/ml and 400 μl of this solution were added to the peptide solution to obtain an MBS concentration of about 5 mg/ml. This solution was stirred constantly at room temperature for 30 minutes. 6 μl of β -mercaptoethanol were added for a final β -mercaptoethanol concentration of 35 mM. The solution was stirred constantly at room temperature for 1 hour. KLH was dissolved in PBS at 30 3 mg/ml and 2.5 ml were added to the peptide solution. The solution was stirred constantly at room temperature for 3 hours and then dialysed against 1 litre of PBS, with three changes of the PBS. The final peptide concentration was about 1 mg/ml and the final KLH concentration was about 1.5 mg/ml.

ANTIBODY GENERATION

Rabbits were injected with the synthetic peptide solutions as follows. 250 μ l each of the glutaraldehyde- and EDC-coupled peptide solutions were together mixed with 500 μ l of Freund's adjuvant. This solution was injected intramuscularly into the rear legs of a rabbit, 500 μ l per leg. The total amount of injected peptide was 0.5 mg. 500 μ l of the synthetic peptide coupled to KLH with MBS were mixed with 500 μ l of Freund's adjuvant. This solution was injected intramuscularly into the rear legs of another rabbit, 500 μ l per leg. The total amount of injected peptide was 0.5 mg.

The synthetic peptide was loaded onto two lanes, 1.5 μ g and 4 μ g, of a gel (18% running, 5% stacking). The gel was blotted overnight at 30V and blocked with 3% milk in PBS. The gel was incubated overnight with rabbit serum diluted 1:250 in 1% milk/PBS followed by incubation with goat anti-rabbit-alkaline phosphatase diluted 1:1000 for 1 hour. The gel was then developed with substrate. The synthetic peptide was seen by comasie blue staining. The peptide was detected by the second bleed of each rabbit and was not detected by the preimmune serum of either rabbit.

Interaction between immobilized peptide and serum antibodies was further studied through surface plasmon resonance using BIAcore™. The synthetic peptide was covalently immobilized on a dextran matrix by amine coupling. Rabbit serum of different dilutions were injected over the surface for five minutes and the amount of antibody bound to the immobilized peptide determined. The titer is defined as the last dilution of the serum giving a positive response, that is, greater than 50 Resonance Units. Using this approach, antibodies were found to be present in serum from both rabbits and the interaction can be blocked by preincubating the serum with the peptide. Antibodies in serum of the rabbits were found not to interact with an immobilized unrelated peptide.

EXPERIMENTS INVOLVING RATS AND ANTIBODIES TO THE CHEMICALLY SYNTHESIZED PEPTIDE

Antibody serum was prepared in 10 mM Tris.Cl at pH 7.4. Each of five rats received 100 μ l of the solution by injection into the left gluteus maximus. Each rat of a second group of five rats was treated similarly, but with an additional injection of solution containing 45 μ g of the polypeptide (SEQ ID NO:1) into the right gluteus maximus. Each rat of a third group of five rats received an injection of 100 μ l of 10 mM Tris.Cl at pH 7.0.

Each of the fifteen rats was then injected as before with tetracycline hydrochloride, in the amount of 24 mg per Kg of body weight. A second dose of tetracycline hydrochloride was injected about 48 hours later. The rats were sacrificed after about another 24 hours.

The bone mineral apposition rate was determined by measurements, described above, of the lower right femoral metaphysis. The results are given in Table Five and Figure 5.

TABLE FIVE: Bone Mineral Apposition Rates in Rats Injected with Antibody to the Chemically Synthesized Peptide			
	Group A	Group B	Group C
Mean ($\mu\text{m/day}$)	0.86	1.22	1.30
S.D.	0.02	0.08	0.11
N	5	5	5
	t	d.f	p
Group A vs Group B	8.06	8	> 0.2
Group A vs Group C	7.57	8	< 0.001
Group B vs Group C	1.24	8	> 0.2

- 5 Methodology and products can be thus be developed using antibody to the polypeptide for use in detecting the polypeptide with which the antibody binds. For example, antibody can be linked to or conjugated with any of several well known reporter systems set up to indicate positively binding of the polypeptide to the antibody. Well known reporter systems include radioimmuno-assays (RIAs) or immunoradiometric assays (IRMAs).
- 10 Alternatively, an enzyme-linked immunosorbent assay (ELISA) would have in common with RIAs and IRMAs a relatively high degree of sensitivity, but would generally not rely upon the use of radioisotopes. A visually detectable substance may be produced or at least one detectable in a spectrophotometer. An assay relying upon fluorescence of a substance bound by the enzyme being assayed could be used. It will be appreciated that there are a
- 15 number of reporter systems which may be used, according to the present invention, to detect the presence of a particular polypeptide. With standardized sample collection and treatment, polypeptide presence above a threshold amount in blood serum could well be determined.

- Such a method based on antigenic response to the chemically synthesized human polypeptide (SEQ ID NO:1) could be developed and variants of the polypeptide
- 20 obtained, as described above for amino acid substitution, deletion and addition, (and conjugates) could then be pre-screened as potential bone stimulating factors. Those that react positively with the antibody to the already known peptide could then be tested for bone stimulatory effects in vivo using the system described herein for rats, for example.
- Such an antibody-linked reporter system could be used in a method for determining whether
- 25 blood serum of a subject contains a deficient amount of the polypeptide. Given a normal

threshold concentration of such a polypeptide in blood serum of a given type of subject, test kits could thus be developed.

EXPERIMENTS INVOLVING CHEMICALLY SYNTHESIZED HUMAN POLYPEPTIDE CONTAINING CYSTEINE→ALANINE SUBSTITUTION

- 5 A modified sequence (SEQ ID NO:3) of the chemically synthesized peptide (SEQ ID NO:1) obtained by substitution of the cysteine residue at position 13 by alanine was prepared by standard chemical procedures. An alanine residue is sterically similar to a reduced cysteine residue while rendering the polypeptide incapable of spontaneous dimerization. A tricine SDS electrophoretic gel of the modified and unmodified (normal) peptides is shown in Figure 6.
- 10 Experiments were carried out on three groups of six rats weighing between 295 and 320 g. A 1 mg per ml solution of the modified peptide (SEQ ID NO:3) was prepared in 0.1% acetic acid. A 1 mg per ml solution of the normal peptide (SEQ ID NO:1) was prepared in 0.1% acetic acid. Each rat of a first of the groups had subcutaneously injected
- 15 into its right thigh 0.1 ml of the modified peptide solution. Similarly, each rat of the second group was injected with 0.1 ml of the normal peptide solution. Each rat of the third group, the control group, was injected with 0.1 ml of 0.1% acetic acid solution. Immediately following these injections, each rat was injected intramuscularly with 24 mg per Kg body weight of tetracycline hydrochloride dissolved in 0.5 ml of water. A second dose of tetracycline
- 20 hydrochloride was administered 48 hours later. The animals were sacrificed 24 hours after the second dose by CO₂ narcosis. The lower metaphysis of the right femur was dissected out and fixed in a 10% aqueous solution of formaldehyde buffered at pH 7.2 by acetate buffer. Bone sections were prepared for measurement as described above.
- 25 The results are tabulated in Table Six and graphically depicted in Figure 7. As can be seen, the bone apposition rate for rats injected with the modified polypeptide is significantly greater than that for those of the control group but below the bone apposition rate shown for the rats injected with the normal peptide.

TABLE SIX: Comparison of the Group Arithmetic Means Among Groups Injected with Modified Peptide, Unmodified Peptide and Control			
	Group A	Group B	Control Group
Mean	1.67 $\mu\text{m/d}$	1.35 $\mu\text{m/d}$	1.02 $\mu\text{m/d}$
S.D.	0.11 $\mu\text{m/d}$	0.16 $\mu\text{m/d}$	0.010 $\mu\text{m/d}$
N	6	6	6
	t	d.f	p
Group A vs Control (Group C)	12.2	10	<0.001
Group B vs Control (Group C)	4.69	10	<0.001
Group A vs Group B	3.97	10	<0.005

EXPERIMENTS INVOLVING ACTIVE FRAGMENTS OF THE 36-AMINO ACID HUMAN POLYPEPTIDE

- Polypeptides having the amino acid sequences identified as SEQ ID NOs:4, 5, 6, 7, 8 and 9 were synthesized according to well known chemical procedures.

- Sprague-Dawley rats were used as test animals to determine bone mineral apposition rate, as described above. Male rats having weights between 280 and 380 g were subject to subcutaneous injection after one week of acclimatization. Each animal was injected with 200 μl of a 0.1% acetic acid test solution, solutions having been prepared at concentrations to obtain a dosage of about 25 nmol of polypeptide per animal. Each test dose was immediately followed by intramuscular injection of 24 mg per Kg of body weight of tetracycline hydrochloride. A second injection of tetracycline was made 48 hours later.

Control Group: 0.1% acetic acid solution

Group A: SEQ ID NO:1:

- Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn Gln Pro

Group E: SEQ ID NO:4:

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val

- Group D: SEQ ID NO:5:

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala

Group C: SEQ ID NO:6:

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu

Group B: SEQ ID NO:7:

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile

- 5 In a similar but separate set of experiments, bone mineral apposition rates were tested using the following chemically synthesized polypeptides:

Group F: SEQ ID NO:8:

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys

Group G: SEQ ID NO:9:

- 10 Arg Thr Asn Glu His Thr Ala Asp Cys Lys

Bone mineral apposition rates were determined by measurements of the lower metaphysis of the right femur, as described previously. Results obtained in the two sets of experiments are summarized in Tables Seven and Eight and graphically depicted in

- 15 Figures 8 and 9. As can be seen, all of the polypeptides tested had a positive effect on bone apposition rate, i.e, displayed bone stimulatory activity.

TABLE SEVEN: Comparison of the Group Arithmetic Means Among First Group Injected with Active Variants						
	Group A	Group B	Group C	Group D	Group E	Control
Mean	1.4	1.41	1.37	1.35	1.31	1.03
S.D.	0.05	0.08	0.09	0.1	0.06	0.06
N	6	6	6	6	6	6
	t		d.f.		p	
Group A vs Control	5.18		10		<0.001	
Group B vs Control	9.67		10		<0.001	
Group C vs Control	7.64		10		<0.001	
Group D vs Control	6.92		10		<0.001	
Group E vs Control	7.99		10		<0.001	
Group A vs Group B	0.14		10		>0.5	
Group A vs Group C	0.4		10		>0.5	
Group A vs Group D	0.66		10		>0.5	
Group A vs Group E	1.3		10		>0.2	
Group B vs Group C	0.82		10		>0.4	
Group B vs Group D	1.19		10		>0.2	
Group B vs Group E	2.49		10		<0.05	

TABLE EIGHT: Comparison of the Group Arithmetic Means Among Second Groups Injected with Active Variants			
	Group F	Group G	Control Group
Mean	2.09 $\mu\text{m/d}$	2.83 $\mu\text{m/d}$	1.63 $\mu\text{m/d}$
S.D.	0.34 $\mu\text{m/d}$	0.19 $\mu\text{m/d}$	0.13 $\mu\text{m/d}$
N	4	3	4
	t	d.f	p
Group F vs Control		6	0.047
Group G vs Control		5	0.0002
Group F vs Group G		5	0.215

BONE CALCIUM CONTENT EXPERIMENTS INVOLVING SEQ ID NO:7

5 A further set of experiments was conducted using the polypeptide identified as SEQ ID NO:7 to determine the effect of the polypeptide on bone calcium content when administered to rats.

10 Ovariectomies were performed on rats as described above. A 0.1% acetic acid solution containing 25 nmoles of the polypeptide was administered subcutaneously to each rat each day for the duration of the experiment. One group of rats was treated for 12 weeks beginning 100 days after ovariectomization. Another group of rats was treated for eight weeks beginning eight weeks after ovariectomization. Rats were sacrificed at the end of the treatment period and dissected and post mortem assessment of bone mineral content was carried out.

15 The lumbar spines L1 - L4 were cleaned with a power nylon brush to remove the attached muscle. They were placed ventral side down under 3 cm of distilled water in a polypropylene container and scanned by a dual energy x-ray absorptometer (DEXA), Hologic 100, to determine the calcium content in grams. The right femur of each rat was also dissected out intact and cleared of the attached muscles with a power nylon brush. It was scanned dorsal side down under 3 cm of distilled water by DEXA. Four regions of the femur were scanned, as indicated in Figures 10 and 11: A, proximal end; B, diaphysis; C, distal end; and D, neck. The bone mineral (i.e., calcium) content in grams was estimated in the four zones of the femur based on absorption and using an internal standard of the machine. Results are tabulated in Tables Nine to Eighteen.

TABLE NINE: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 100 Days to Ovariectomized Rats--Bone Mineral Content Measured in Proximal End of Femur			
	Control	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.1503	0.1351	0.1411
S.D.	0.0159	0.0105	0.0155
N	14	14	11
	t	d.f	p
Control vs A	2.9772	26	<0.025
Control vs B	1.44	23	N.S.
Group A vs Group B	1.1634	23	N.S.

TABLE TEN: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 56 Days to Ovariectomized Rats--Bone Mineral Content Measured in Proximal End of Femur				
	Control	Sham	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.1451	0.1387	0.1368	0.1328
S.D.	0.0183	0.0166	0.028	0.0141
N	5	5	6	6
	t		d.f	p
Control vs Sham	0.7372		8	N.S.
Control vs A	0.6261		9	N.S.
Control vs B	1.6223		9	N.S.
Sham vs A	0.133		9	N.S.
Sham vs B	1.6229		9	N.S.
Group A vs B	0.3116		10	N.S.

TABLE ELEVEN: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 100 Days to Ovariectomized Rats--Bone Mineral Content Measured in Spine (L1-L4)			
	Control	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g)	0.5437	0.4364	0.4758
S.D.	0.0161	0.0089	0.0188
N	14	14	10
	t	d.f	p
Control vs A	5.8384	26	<0.001
Control vs B	2.7434	22	<0.0025
Group A vs Group B	2.0756	22	0.05

TABLE TWELVE: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 56 Days to Ovariectomized Rats--Bone Mineral Content Measured in Spine (L1-L4)				
	Control	Sham	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.5542	0.5321	0.4322	0.4606
S.D.	0.0275	0.0172	0.0226	0.0234
N	5	5	6	6
	t		d.f	p
Control vs Sham	0.6805		8	N.S.
Control vs A	4.4196		9	<0.005
Control vs B	3.1042		9	<0.025
Sham vs A	2.8382		9	<0.025
Sham vs B	1.9951		9	N.S.
Group A vs B	0.8759		10	N.S.

TABLE THIRTEEN: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 100 Days to Ovariectomized Rats--Bone Mineral Content Measured in Femoral Diaphysis

	Control	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.2258	0.2146	0.2347
S.D.	0.0261	0.0106	0.0215
N	14	14	11
	t	d.f	p
Control vs A	0.8301	26	N.S.
Control vs B	0.9078	23	N.S.
Group A vs Group B	2.3079	23	<0.05

TABLE FOURTEEN: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 56 Days to Ovariectomized Rats--Bone Mineral Content Measured in Femoral Diaphysis

	Control	Sham	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.2179	0.1918	0.1716	0.2091
S.D.	0.0156	0.0162	0.0272	0.0121
N	5	5	6	6
	t		d.f	p
Control vs Sham	2.259		8	<0.05
Control vs A	3.3549		9	<0.025
Control vs B	1.9209		9	N.S.
Sham vs A	1.4571		9	N.S.
Sham vs B	1.1778		9	N.S.
Group A vs B	2.4926		10	<0.05

TABLE FIFTEEN: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 100 Days to Ovariectomized Rats-Bone Mineral Content Measured in Distal End of Femur			
	Control	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.1597	0.1396	0.1424
S.D.	0.0185	0.0068	0.0132
N	14	14	11
	t	d.f	p
Control vs A	3.8255	26	<0.001
Control vs B	2.616	23	<0.025
Group A vs Group B	0.6984	23	N.S.

TABLE SIXTEEN: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 56 Days to Ovariectomized Rats-Bone Mineral Content Measured in Distal End of Femur				
	Control	Sham	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.1826	0.154	0.1304	0.1347
S.D.	0.0122	0.0118	0.0094	0.0039
N	5	5	6	6
	t		d.f	p
Control vs Sham	3.7549		8	<0.025
Control vs A	8.0183		9	<0.001
Control vs B	9.1462		9	<0.001
Sham vs A	3.7046		9	<0.005
Sham vs B	3.8149		9	<0.005
Group A vs B	1.0274		10	N.S.

TABLE SEVENTEEN: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 100 Days to Ovariectomized Rats--Bone Mineral Content Measured in Femoral Neck

	Control	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.0334	0.0303	0.0351
S.D.	0.0049	0.004	0.0031
N	14	14	10
	t	d.f	p
Control vs A	1.3978	26	N.S.
Control vs B	1.0326	21	N.S.
Group A vs Group B	2.259	21	P<0.005

TABLE EIGHTEEN: Comparison of Group Arithmetic Means Among Groups Injected with Polypeptide SEQ ID NO:7 Administered over 56 Days to Ovariectomized Rats--Bone Mineral Content Measured in Femoral Neck

	Control	Sham	A-Ovariectomized (no polypeptide)	B-Ovariectomized (with polypeptide)
Mean (g.)	0.0277	0.0255	0.0202	0.0274
S.D.	0.002	0.0038	0.0028	0.0013
N	5	5	6	6
	t	d.f	p	
Control vs Sham	1.1534	8	N.S.	
Control vs A	4.9809	9	<0.001	
Control vs B	0.3342	9	N.S.	
Sham vs A	2.662	9	<0.05	
Sham vs B	1.1462	9	N.S.	
Group A vs B	5.6713	10	<0.005	

As can be seen from the tabulated data, the increase in *in vivo* calcium bone content is most obvious in the femoral neck and femoral diaphysis, implying that the effect of the administered peptide can be site specific, possibly being greater at skeletal sites under mechanical stress.

5 EXPERIMENTS INVOLVING OTHER FRAGMENTS OF THE 36-AMINO ACID HUMAN POLYPEPTIDE

Polypeptide fragments of the normal polypeptide (SEQ ID NO:1) were also synthesized and tested for bone stimulatory activity as with the C-terminus fragments.

Control Group: 0.1% acetic acid

Group H: SEQ ID NO:1:

- 10 Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys
Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn Gln Pro

Group I: SEQ ID NO:16:

Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile

Group J: SEQ ID NO:15:

- 15 Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr
Leu Met Val Leu Asp Gln Asn

Group K: SEQ ID NO:14:

Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu
Asp

- 20 Group L: SEQ ID NOs: 10,11,12 & 13 (mixture):

Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn Gln
Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn
Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln
Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp

- 25 Bone mineral apposition rates were again determined by measurement of the lower metaphysis of the right femur. Results obtained are summarized in Table Nineteen and graphically depicted in Figure 12. As can be seen in Figure 12, none of the non-N-terminus variants identified as SEQ ID NO: 10, 11, 12, 13, 14, 15 or 16 was found to increase the bone apposition rate with respect to the control.

TABLE NINETEEN: Summary of the Group Arithmetic Means for Bone Apposition Rates of Rats Injected with Non-N-terminus Variants						
	Group H	Group I	Group J	Group K	Group L	Control
Mean ($\mu\text{m/day}$)	1.5	1.02	0.92	0.92	0.98	1.02
S.D.	0.09	0.12	0.09	0.04	0.09	0.06
N	6	6	6	6	6	6

- A polypeptide having the ten amino acid sequence of SEQ ID NO:9, but protected at both ends was synthesized and tested for bone stimulatory activity in comparison to polypeptides identified as SEQ ID NOs: 1, 7 and 9. The protected polypeptide was
- 5 acetylated at the amino terminus and amidated at the carboxy terminus and is identified herein as SEQ ID NO:24. Results obtained according to experimental procedures described above, using about 125 nmoles of polypeptide per Kg of body weight of animal are presented in Table Twenty and Figure 13.

TABLE TWENTY: Comparison of the Mean Bone Apposition Rates Among Rats Treated with SEQ ID NOs: 1, 7, 9 and 24					
	SEQ ID NO:1	SEQ ID NO:7	SEQ ID NO:9	SEQ ID NO:24	Control
Mean ($\mu\text{m/day}$)	2.03	2.18	2.11	2.72	1.7
S.D.	0.1	0.26	0.18	0.15	0.28
N	6	6	6	6	6

	t	d.f.	p
SEQ ID NO:1 vs Control	>2.6	10	<0.025
SEQ ID NO:7 vs Control	-3.03	10	<0.003
SEQ ID NO:9 vs Control	-2.96	10	<0.006
SEQ ID NO:24 vs Control	-7.73	10	<0.00
SEQ ID NO:1 vs 7	-1.28	10	>0.2
SEQ ID NO:1 vs 9	-0.898	10	>0.3
SEQ ID NO: vs 24	-4.42	10	<0.002
SEQ ID NO:7 vs 9	0.548	10	>0.5
SEQ ID NO:7 vs 24	-4.42	10	<0.002
SEQ ID NO:9 vs 24	-6.38	10	<0.00

There are literature reports that the presence of histidine and cysteine residues in polypeptides can effect degradation of asparaginy- and aspartyl-containing polypeptides in the absence of catalytic enzymes [Int. J. Peptide Protein Res. 45, 1995, 547,553]. The following analogues of the polypeptide identified as SEQ ID NO:9 were synthesized:

SEQ ID NO:25

CH₃CO - Arg Thr Asn Glu His Thr Ala Glu Cys Lys - NH₂

SEQ ID NO:26

10 CH₃CO - Arg Thr Gln Glu His Thr Ala Glu Cys Lys - NH₂

SEQ ID NO:27

CH₃CO - Arg Thr Gln Glu His Thr Ala Asp Cys Lys - NH₂

Each of the polypeptides having the sequences identified as SEQ ID NO: 7 and 24 was dissolved in 5 mM acetic acid to a final concentration of 1mg/ml and incubated at 15 37°C. The peptide compositions were analyzed weekly by capillary electrophoresis on a P/ACE 6000 system (Beckman) using 50 mM sodium phosphate pH 2.5 as the running buffer on a 57 cm long x 75 µm internal diameter capillary. The incubated peptide (20µl) was diluted with 80 µl of running buffer, placed in a 500 µl vial prior to injection onto the P/ACE using pressure for 20 seconds. Following electrophoresis of the incubated peptide at 30 kV for 15 20 min a second run was carried out using freshly dissolved peptide as a control.

Analytical results indicated that each of the polypeptides tested underwent modification. Mass spectroscopic results (not shown) indicated that both peptides were breaking down to smaller fragments, i.e., undergoing proteolysis.

Each of the polypeptides was dissolved in 20 mM sodium phosphate pH 3.0, 25 20 mM ammonium acetate pH 4.0, 20 mM ammonium acetate pH 5.0, 20 mM MES pH 6.0, 20 mM sodium phosphate pH 7.0, 20 mM sodium phosphate pH 7.5, 20 mM sodium phosphate pH 8.0, 20 mM ammonium acetate pH 8.5 or 20 mM ammonium acetate pH 9.5 to a final concentration of 1 mg/ml. The peptide was incubated at 37°C and the peptide assayed weekly by separation on P/ACE as described above. Some samples were separated by RP- 30 HPLC and the isolated peaks subjected to mass spectroscopic analysis.

Analytic results indicated that the polypeptide identified as SEQ ID NO: 24 was most stable at a pH near 4.5. When incubated above pH 6.0, the peptide dimerized. The peptide degraded when incubated below pH 4.0. The polypeptide having the amino acid sequence identified as SEQ ID NO:7 had a similar stability profile.

35 The protected polypeptide identified as SEQ ID NO:24 was dissolved in 20 mM or 250 mM ammonium acetate pH 4.5 to a final concentration of 1 mg/ml. In some experiments the buffer was supplemented with 20 mM EDTA. The peptide solution was then incubated at -70°C, -20°C, 4°C or room temperature (22°C). Samples were assayed weekly by electrophoresis on the P/ACE as described above and/or by RP-HPLC.

Analytical results indicated that the peptide is very stable as a powder and when dissolved in pH 4.5 buffer. Modification of the peptide incubated at room temperature was observed after 7 days as a peak eluting before the intact peptide on the RP-HPLC chromatogram. The peptide modification was not altered by addition of 20 mM EDTA or by
5 incubation in 250 mM ammonium acetate. Dissolved peptide incubated at 4°C, -20°C or -70°C was unchanged as compared with dry peptide stored at -70°C. After 14 days of incubation at room temperature, the HPLC profile included the two peaks observed at day 7, plus an additional peak with a retention time greater than intact peptide. While the dissolved peptide incubated at room temperature continued to become modified with increasing time, it
10 was apparent that it was most stable in the lower salt buffer with the addition of 20 mM EDTA. Incubation of peptide at all other temperatures did not significantly alter the HPLC profile as compared with dry peptide stored at -70°C up to 28 days.

The stability of each of the polypeptides having the amino acid sequences identified as SEQ ID NOs: 25, 26 and 27 was tested as described above.

15 The polypeptide having SEQ ID NO: 27 was found to be unstable at all pHs tested when incubated at 37°C for 3 days. Numerous additional peaks were observed on its electropherogram. The polypeptide having SEQ ID NO:25 was stable at pH 2.5 - 3.0 for 20 days. The polypeptide having SEQ ID NO:26 was found to be stable at pH 2.5 - 3.0 when incubated at 37°C.

20 In general, polypeptides having amino acid sequences identified as SEQ ID NOs: 7 and 24 degraded over time when dissolved in dilute acids. These peptides were found to be most stable when dissolved in pH 4.5 buffer. Analogues, SEQ ID NOs: 25 and 26 were found to be more stable than SEQ ID NOs: 7 or 24, while SEQ ID NO:27 was found to be significantly less stable.

25 Polypeptides having the amino acid sequences identified as SEQ ID NOs:25, 26 and 27 were tested for bone stimulatory activity in comparison to the polypeptide identified as SEQ ID NOs: 24. Results obtained according to experimental procedures described above are presented in Table Twenty-One and Figure 14. Polypeptides having the amino acid sequences identified as SEQ ID Nos:26, 44, 45 and 46 were tested for bone stimulatory
30 activity in a series of experiments, again according to experimental procedures described above using approximately 500 gm male Sprague-Dawley rats and 300 nmol/kg bodyweight of test animal. Results are presented in Table Twenty-Two and Figure 15.

TABLE TWENTY-ONE: Comparison of the Mean Bone Apposition Rates Among Rats Treated with SEQ ID NOs: 24, 25, 26 and 27					
	SEQ ID NO:24	SEQ ID NO:25	SEQ ID NO:26	SEQ ID NO:27	Control
Mean (µm/day)	2.04	1.94	2.88	1.72	1.36
S.D.	0.14	0.23	0.47	0.33	0.14
N	4	4	4	4	4

TABLE TWENTY-TWO: Comparison of the Mean Bone Apposition Rates Among Rats Treated with SEQ ID NOs: 26, 44, 45 and 46					
	SEQ ID NO:26	SEQ ID NO:44	SEQ ID NO:45	SEQ ID NO:46	Control
Mean (µm/day)	2.04	1.66	1.77	2.31	1.34
S.D.	0.09	0.21	0.13	0.21	0.07
N	4	4	4	4	4

- 5 A general charge pattern, based on the side chains of the component amino acids, is shared by the 10-amino sequences identified as SEQ ID NOs:9, 24, 25 26 and 27:



- 10 In the case of each side chain indicated by an "X", the side chain would not bear a full ionic charge under physiological conditions. It is known, to a person skilled in the art, that the side chain of threonine (second and sixth amino acids) includes a hydroxyl group, which is polar. Asparagine, the third amino acid, is also polar. Alanine, the seventh amino acid is considered to be relatively non-polar. Cysteine, the ninth amino acid, is considered polar, but the polypeptide is also thought to spontaneously dimerize by formation of an intermolecular disulfide bridge, as described above.
- 15 In one set of experiments, a series of polypeptides having amino acid sequences identified as SEQ NOs:34, 35, 36, 37 and 38 were chemically synthesized according to routine procedures and effects on bone mineral apposition rate in rats tested. Each of these polypeptides has the same amino acid sequence as that identified as SEQ ID NO:24, but one of the amino acids having a side chain bearing a charge has been substituted
- 20 by the amino acid alanine. In each of these tests, four rats (about 300 grams) were tested for each compound. 100 nmoles of material (in 400 µl 20 mM acetic acid solution) was injected

subcutaneously into each animal along with tetracycline (5 mg per animal in 400 µl water), as described above. A second dosage of tetracycline was administered 48 hours later and the animals sacrificed 24 hours after that. The lower metaphysis of the right femur was examined to determine the bone mineral apposition rate. For comparison, experiments were performed using a control in which no compound was present in the acetic acid solution and using a polypeptide having the sequence identified as SEQ ID NO:24. In all cases the N-terminus of the test compound was acetylated and C-terminus was amidated. The results obtained are shown in Figure 15.

In another set of experiments, the dosage dependence of effects observed with the series of compounds having amino acid sequences identified as SEQ NOs:34, 35, 36, 37 and 38 was examined. The experiments were carried out as described above for these polypeptides for three dosages of each polypeptide: 100, 200 and 400 nmoles per animal. The results obtained are shown in Figure 16.

As can be seen from Figures 15, 16 and 17, substitution of any of the first, fourth, fifth, eighth or tenth amino acids of SEQ ID NO:24 with alanine, results in a substantial loss of bone stimulatory activity. On the other hand, substitution of any of the second, third, sixth or seventh amino acids of SEQ ID NO:24 largely results in retention of bone stimulatory activity.

In a final set of experiments, a polypeptide having the ninth amino acid, cysteine, replaced by the amino acid tyrosine, SEQ ID NO:43, was synthesized and tested for bone stimulatory activity. Four rats (about 300 grams) were each administered with 100 nmoles of material (in 400 µl 20 mM acetic acid solution) by subcutaneous injection along with tetracycline (5 mg per animal in 400 µl water), as described above. A second dosage of tetracycline was administered 48 hours later and the animals sacrificed 24 hours after that. The lower metaphysis of the right femur was examined to determine the bone mineral apposition rate. For comparison, experiments were performed using a control in which no compound was present in the acetic acid solution. The results obtained are shown in Figure 18.

As can be seen from Figure 18, a modest increase in bone stimulatory activity was observed in animals to which the polypeptide having SEQ ID NO:43 was administered over the control group.

Included within the scope of the present invention are compounds derived from the polypeptide having the amino acid sequence identified as SEQ ID NO:9. Included within this family of compounds are those polypeptides having SEQ ID NOs:24, 25, 26, 27, 39, 40, 42 and 42. Such a polypeptide can have up to or be based on 30, 25, 20, 15 or 10 consecutive amino acids from the amino acid sequence identified as SEQ ID NO:1.

A compound "derived from" a polypeptide having a particular amino acid sequence is any molecular entity which is identical, substantially homologous, or otherwise functionally or structurally equivalent to that polypeptide. Thus, a molecule derived from a

particular polypeptide may encompass the amino acid sequence of the polypeptide, any portion of that polypeptide, or other molecular entity that functions to stimulate bone growth. A molecule derived from such a binding domain will mimic the polypeptide from which it is derived. Such molecular entities may include peptide mimetics and the like.

5 "Peptides mimetics" are structures which serve as substitutes for peptides in interactions with acceptor molecules (see Morgan et al. (1989) Ann. Reports Med. Chem. 24:243-252 for a review of peptide mimetics). Peptide mimetics, as used herein, include synthetic structures which may or may not contain amino acids and/or peptide bonds, but retain structural and functional features of a peptide from which they are derived. The term, 10 "peptide mimetics" also includes peptoid and oligopeptoids, which are peptides or oligomers of N-substituted amino acids (Simon et al. (1972) Proc. Natl. Acad. Sci USA 89:9367-9371). Further included as peptide mimetics are peptide libraries, which are collections of peptides designed to be a given amino acid length and representing all conceivable sequences of amino acids corresponding thereto.

15 Two polypeptide sequences are "substantially homologous" when at least about 85% (preferably at least about 85% to 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the polypeptide. As used herein, substantially homologous also refers to sequences showing identity to the specified polypeptide sequence.

20 Peptide mimetics which structurally and functionally mimic the polypeptides having bone stimulatory activity described herein, will also find use herein and may be generated using the following strategies and procedures. Generally, mimetics are designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chain moieties by a methyl group or pseudoisosteric groups with different 25 electronic properties (see Hruby et al. (1990) Biochem, J. 268:249-262), and by systematic replacement of peptide bonds in the above described peptide inhibitors with amide bond replacements. For example, analogues containing amide bond surrogates may be used to investigate aspects of peptide structure and function, such as rotational freedom in the backbone, intra and intermolecular hydrogen-bond patterns, modifications of local and total 30 polarity and hydrophobicity, and oral bioavailability.

Local conformational constraints can also be introduced to determine conformational requirements for activity of a potential peptide mimetic having bone stimulatory activity. For example, β,β -distributed amino acids may be used to examine the effects of conformational constraints on peptide activity (see, e.g. Manning et al. (1982) 35 J. Med. Chem. 25:408-414; Mosberg et al. (1983) Proc. Natl. Acad. Sci. USA 106:506-512; Pelton et al. (1985) Proc. Natl. Acad. Sci. USA 82:236-239).

The mimetics can include isosteric amide bonds such as $\psi[\text{CH}_2\text{S}]$, $\psi[\text{CH}_2\text{NH}]$, $\psi[\text{CNH}_2]$, $\psi[\text{NHCO}]$, $\psi[\text{COCH}_2]$ and $\psi[(\text{E}) \text{ or } (\text{Z}) \text{CH}=\text{CH}]$ see, for review, Spatola (1983) in "Chemistry and Biochemistry of Amino Acids, Peptides and Proteins," Volume VII. (Weinstein

ed.), Marcel Dekker, New York, 267-357). The synthetic molecules can also include D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule from enzymatic degradation (see, e.g. Freidinger et al (1985) in "Peptides: Structure and Function." (Deber et al. eds.), Pierce Chem Co., Rockford, Ill., 549-552; Sawyer et al (1980) Proc. Natl. Acad. Sci. USA 77:5754-5758; Torchiana et al (1978) Arch. Int. Pharmacol. Ther. 235:170-176). Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states, e.g. $\alpha\alpha'$ - and $\beta\beta$ -substituted cyclic amino acids such as 1-aminocyclopentanecarboxylic acid (cycloleucine) and β,β -cyclopentamethylene- β -mercaptopropionic acid (see Hruby et al (1990), *supra*).

- 10 The mimetics can also include mimics of polypeptide secondary structure—structures which can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins—including β -turn mimetics, such as phenoxathin ring system, and β -sheet mimics, such as epindolidione structures. Design synthesis and conformational analysis of a α -helix inducing template has been described (Kemp et al (1988) *Tetrahedron Lett.* 29:4931; Kemp et al. (1988) *Tetrahedron Lett.* 29:4935).

- 15 A potential mimetic can be tested, or pre-screened, for potential activity as a bone stimulating compound by measuring the affinity of the compound for an antibody raised against the polypeptide from which the mimetic is derived. As described above for polypeptides, those mimetics that react positively with the antibody to the already known peptide could then be tested for bone stimulatory effects *in vivo* using the system described herein for rats, for example. Antibodies raised against a polypeptide having the amino acid sequence identified as SEQ ID NO:9 would be particularly useful in this context.

- 20 Peptoids will find use herein. Peptoids are oligomers of N-substituted amino acids (Simon et al (1972), *supra*). and can be used as motifs for the generation of chemically diverse libraries of novel molecules, which can then be tested for binding and bone stimulatory activity. The monomers may incorporate t-butyl-based side-chain and 9-fluorenylmethoxy-carbonyl α -amine protection. Oligomerization of the peptoid monomers may be performed by for example, *in situ* activation by either benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate or bromotris(pyrrolidino)phosphonium hexafluorophosphate. Other steps are identical to conventional peptide synthesis using α -(9-fluorenylmethoxycarbonyl)amino acids. Oligopeptoids may be identified which have affinities comparable to the corresponding polypeptides and, thus, are potentially useful as bone stimulatory agents.

- 30 A compound or polypeptide having the "charge pattern" of a particular polypeptide has the number and distribution (i.e., same order) of the charges of the side chains of the amino acids of the sequence of the particular polypeptide. The charge of each side chain is based on the predominant charge that is present under physiological conditions. Spacing of the charges would also be similar to that provided by the polypeptide. In preferred

instances, the spacing would be substantially the same as that provided by the polyamide backbone of the particular polypeptide, and so the compound is said in such instances to have substantially the same "charge pattern and spacing" of the polypeptide.

5 A summary of the results obtained with respect to particular polypeptide sequences tested is provided in Figure 19.

As can be seen, the polypeptide identified as SEQ ID NO:24 (and the corresponding unprotected polypeptide, SEQ ID NO:9) has a sequence of 10 amino acids contained in the 36 amino acid sequence of the polypeptide identified as SEQ ID NO:1. Thus, in vivo bone stimulatory activity can be retained with a polypeptide in which as little as 10 28% of the amino acid sequence of SEQ ID NO:1 is conserved. In particular, the protected version of the 10-amino acid polypeptide sequence, SEQ ID NO:24, has a bone stimulatory effect which exceeds that of either SEQ ID NO:1 or the 10-amino acid unprotected version, SEQ ID NO:9.

Further, As can be seen the polypeptide identified as SEQ ID NO:26 (and the 15 corresponding unprotected polypeptide, SEQ ID NO:29) has a sequence of 10 amino acids, only eight of which are identical to those contained in the 36 amino acid sequence of the polypeptide identified as SEQ ID NO:1. This indicates that in vivo bone stimulatory activity can be retained in a polypeptide in which as little as 22% of the amino acid sequence of SEQ ID NO:1 is conserved. On a molar basis, the protected sequence (SEQ ID NO:26) has been 20 found, at least under the conditions of the foregoing experiments, have an even more potent effect than that of SEQ ID NO:24.

Likewise, the polypeptide identified as SEQ ID NO:25 (and the corresponding unprotected polypeptide, SEQ ID NO:28) has a sequence of 10 amino acids, only nine of which are identical to those contained in the 36 amino acid sequence of the polypeptide 25 identified as SEQ ID NO:1. This indicates that in vivo bone stimulatory activity can be retained in a polypeptide in which as little as 25% of the amino acid sequence of SEQ ID NO:1 is conserved. On a molar basis, the protected sequence (SEQ ID NO:25) has been found under the conditions of the experiments described above to have a comparable bone stimulatory effect to that of SEQ ID NO:24.

30 The polypeptide identified as SEQ ID NO:27 (and the corresponding unprotected polypeptide, SEQ ID NO:30) has a sequence of 10 amino acids, only nine of which are the same as those contained in SEQ ID NO:1. The protected version of this polypeptide was found to have the bone stimulatory effect also, but it was not as great as that of SEQ ID NO:26.

35 As indicated in the experiments described above, the polypeptides vary from each other according to the conditions to which the polypeptides are exposed. It is generally desirable that an active polypeptide not be degraded to an inactive or less active moiety when stored or during administration. Information about the stability of an active fragment is useful

in formulating preparations for storage and administration. Stability information might also be useful in selecting a fragment that is longer lived once administered to an individual.

Of course it is known to those skilled in the art that polypeptides which provide similar activity are generally related by having the same or similar three-dimensional portion(s) which interacts with another agent, such as a receptor with which the polypeptide binds in some way. This is why it is possible to have several polypeptides that are related to each other that display similar bone-stimulating activity.

The present invention provides a synthetic polypeptide having in vivo bone stimulatory activity in mammals and which increases calcium density or content in bones of mammals, having an amino acid sequence which is at least about 19% conserved in relation to the amino acid sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom, or a homologue thereof. In the context of this invention, a peptide containing an amino acid sequence that can be aligned with that of SEQ ID NO:1 such that at least about 30% of individual amino acid residues of the original sequence are present in the peptide is said to be about 30% conserved with the amino acid sequence identified as SEQ ID NO:1, allowing for homologous substitutions and a limited number of insertions or deletions between aligned sequences. An amino acid sequence having seven out of the 36 amino acid residues of SEQ ID NO:1 in aligned sequence would be 19% conserved. An amino acid sequence having eight out of the 36 amino acid residues of SEQ ID NO:1 in aligned sequence would be 22% conserved, such as is the case of SEQ ID NOs:26 and 29. An amino acid sequence having nine out of the 36 amino acid residues of SEQ ID NO:1 in aligned sequence would be 25% conserved, as for SEQ ID NOs:25, 27, 28 and 30. An amino acid sequence having ten out of the 36 amino acid residues of SEQ ID NO:1 in aligned sequence is 28% conserved, as for SEQ ID NOs:9 and 24.

Described in a slightly different way, a polypeptide of the present invention is an amino acid sequence corresponding to SEQ ID NO:1 with (a) one amino acid to 4 amino acids deleted from the N-terminus of SEQ ID NO:1, (b) one to 22 amino acids deleted from the C-terminus of SEQ ID NO:1, or both (a) and (b); or a functionally equivalent homologue. It may be found possible to delete 5 or 6 or more amino acids from the N-terminus or to delete more than 22 amino acids from the C-terminus of SEQ ID NO:1.

In another sense, the polypeptide of the present invention can be described as a polypeptide exhibiting bone stimulatory activity in mammals, the polypeptide having the sequence identified as SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, or SEQ ID NO:9; analogues thereof wherein the amino acids in the sequence may be substituted, deleted or added, so long as the bone stimulatory activity in mammals derived from the three dimensional structure of the sequence is preserved; and conjugates of each of the polypeptides or analogues thereof, wherein if the polypeptide sequence has that identified as SEQ ID NO:1, then there is at least one amino acid deleted therefrom.

A polypeptide of the present invention would include such a sequence which sequence would have a molecular weight in the range of from about 1000 to 4000. It is to be understood however that the sequence might be added to by conjugation or other technique, which could increase the molecular weight of the overall compound beyond 4000.

5 It will also be understood, without the intention of being limited thereby, that a variety of substitutions of amino acids is possible while "preserving" the three-dimensional structure responsible for the bone stimulatory effect of the polypeptides disclosed herein. It is thus expected, for example, that interchange among non-polar aliphatic neutral amino acids, glycine, alanine, proline, valine and isoleucine, would be possible. Likewise, substitutions
10 among the polar aliphatic neutral amino acids, serine, threonine, methionine, cysteine, asparagine and glutamine could possibly be made. This being said, the linkage of the peptides together by the disulfide bridge appears to be of some importance, and so the lone cysteine residue should probably be held intact and other amino acids capable of forming a disulfide linkage not be substituted elsewhere in the sequence, although as seen above a
15 successful cys→ala substitution was effected (SEQ ID NO:3). Substitutions among the charged acidic amino acids, aspartic acid and glutamic acid, can be made, as shown above, and substitutions among the charged basic amino acids, lysine and arginine are also likely to be possible. Substitutions can be made alone or in combination. These sorts of substitutions and interchanges are well known to those skilled in the art. United States Patent Nos.
20 5,487,983 and 5,512,548, for instance, describes other possible substitutions including substitutions involving amino acids not encoded by the gene. Other substitutions might well be possible.

The importance of the N-terminus portion of the sequence is evident from the results described herein. The polypeptides (SEQ ID NOs:9, 24, 25, 26, 27, 28, 29 and 30)
25 having amino acids 5 to 14 of SEQ ID NO:1 displays bone stimulatory activity while polypeptides lacking the first nine N-terminus amino acids, but having amino acids 10 to 32 (SEQ ID NO:14) or amino acids 20 to 35 (SEQ ID NO:10) do not display bone stimulatory activity. It may be that it is possible to delete more amino acids from either end of the polypeptide identified as SEQ ID NO:9 while retaining the three-dimensional configuration of
30 the subsequence of the polypeptide responsible for bone stimulatory activity. Internal deletions, although they might be possible to some limited extent, should be few. Of particular note, is the polypeptide having the sequence identified as SEQ ID NO:16, which differs by only one amino acid residue from the amino acid sequence identified as SEQ ID NO: 9. The former does not display activity while the latter does display bone stimulatory
35 activity. It is possible using the experimental methods disclosed herein to distinguish between sequences which do and do not stimulate bone growth and which do and do not increase calcium bone content.

It should still be possible for minor additions of amino acids to be made at the ends of the sequence and symmetrical or nearly symmetrical additions to the carboxy and

amino terminals are likely to be possible. Internal additions, although likely to be possible to some limited extent, should be few.

Of the above-listed modifications to the sequence, terminal additions, deletions or substitutions are most likely to be most useful, as such a modification can serve a variety of functions: an identifying group as for use in a radioimmunoassay; or a linking group, as examples.

As with the normal peptide (SEQ ID NO:1), an active subsequence containing a cysteine residue (i.e., SEQ ID Nos: 4, 5, 6, 7, 8 or 9) can spontaneously dimerize and exist in the dimeric form, at least under certain conditions.

Experiments described above also show that it is possible to make a limited number of what would generally be considered "non-conservative" substitutions and still retain bone stimulatory activity. This is particularly true for the amino acid sequence corresponding to SEQ ID NO:9, where for example, it has been shown that with preservation of the charge pattern, the asparagine residue occupying the third position along the sequence can be replaced by alanine.

A further advantage may be obtained through chimeric forms of the protein, as known in the art. A DNA sequence encoding the entire protein, or a portion of the protein, could thus be linked with a sequence coding for the C-terminal portion of E. coli β -galactosidase to produce a fusion protein, for example. An expression system for human respiratory syncytial virus glycoproteins F and G is described in United States Patent No. 5,288,630, issued February 22, 1994, and references cited therein, for example.

A polypeptide of the present invention would usually be synthetic, whether prepared by techniques of conventional "chemistry" or by recombinant techniques. Here, a polypeptide so produced is referred to as being substantially pure or biochemically pure when it is generally free of polypeptides or proteins with which it would occur if found directly in nature, as in blood serum from an animal, for example.

Nucleic acid (DNA) sequences coding for the active portions of the normal polypeptide would be as follows:

SEQ ID NO:17 (corresponding to SEQ ID NO:4):

GGG ATC GGA AAA CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA ATT AAA CCG
AAC ACC TTG CAT AAA AAA GCT GCA GAG ACT TTA ATG GTC

SEQ ID NO:18 (corresponding to SEQ ID NO:5):

GGG ATC GGA AAA CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA ATT AAA CCG
AAC ACC TTG CAT AAA AAA GCT GCA

SEQ ID NO:19 (corresponding to SEQ ID NO:6):

GGG ATC GGA AAA CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA ATT AAA CCG
AAC ACC TTG

SEQ ID NO:20 (corresponding to SEQ ID NO:7):

GGG ATC GGA AAA CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA ATT

SEQ ID NO:21 (corresponding to SEQ ID NO:8):

GGG ATC GGA AAA CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA

5 SEQ ID NO:22 (corresponding to SEQ ID NO:9):

CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA

Hypothetical coding sequences for polypeptides based on SEQ ID NO:1 but having amino acid residues substituted therefor, are:

SEQ ID NO:31 (corresponding to SEQ ID NO:28):

10 CGA ACA AAT GAA CAT ACG GCA GAA TGT AAA

SEQ ID NO:32 (corresponding to SEQ ID NO:29):

CGA ACA CAA GAA CAT ACG GCA GAA TGT AAA

SEQ ID NO:33 (corresponding to SEQ ID NO:30):

CGA ACA CAA GAA CAT ACG GCA GAT TGT AAA

15 Accordingly, a vector incorporating such a DNA sequence could be constructed for use in synthesizing a polypeptide, as described previously, and particularly in international patent application No. PCT/CA 94/00144. The DNA sequence coding for the polypeptide identified as SEQ ID NO:1 is given as SEQ ID NO:23 in the sequence listing of this specification.

20 A DNA sequence or fragment of the present invention may be any fragment that contains a nucleotide sequence which encodes a polypeptide of the present invention. In addition to any of the above coding sequences, the DNA fragment can have an appropriate promoter and an SD sequence (or a suitable ribosome binding site) at its 5'-end, and if necessary, a nucleotide sequence containing a translation initiation codon at the 5'-end and a
25 nucleotide sequence containing a termination codon at the 3'-end.

As known to those skilled in the art, the genetic code is "degenerate". A nucleotide in a gene sequence can thus be replaced by another nucleotide in accordance with the degeneracy of a particular codon (coding triplet), without changing the amino acid sequence of the polypeptide coded for by the gene. A DNA fragment of the present invention
30 can thus be derived from any of the above sequences (and DNA sequences corresponding to substituted polypeptide or other analogues not explicitly illustrated), and such replacement might be done in such a way that the resulting codon(s) shows a high utilization frequency in a specific host cell when producing a polypeptide of the present invention using genetic engineering techniques.

As used herein, "protected" terminal amino group refers to a terminal amino group (N-terminus) coupled with any of various amino-terminal protecting groups that can be employed in peptide synthesis. Examples of suitable groups include acyl protecting groups, for example, formyl, acetyl, benzoyl, trifluoroacetyl, succinyl, and methoxysuccinyl; aromatic urethane protecting groups, for example benzyloxycarbonyl; and aliphatic urethane protecting groups, for example t-butoxycarbonyl or adamantyloxycarbonyl (Gross and Mienhofer, eds., *The Peptides*, vol 3, pp. 3 to 88 (Academic Press, New York, 1981)).

As used herein, "protected" terminal carboxyl group refers to a terminal carboxyl group (C-terminus) coupled with any of various carboxy-terminal protecting groups.

As will be readily apparent to a person skilled in the art, suitable groups include *t*-butyl, benzyl or other acceptable groups linked to the terminal carboxyl group through an ester or ether bond.

Compounds within the scope of this invention can be synthesized chemically by means well known in the art such, for example, solid phase peptide synthesis. The synthesis is commenced from the carboxy-terminal end of the peptide using an α -amino protected amino acid. *t*-Butyloxycarbonyl (Boc) protective groups, or other suitable protective groups, can be used (Stewart et al., "Solid-Phase Peptide Synthesis," W. H. Freeman Co., San Francisco (1969); Merrifield, *J. Am. Chem. Soc.* 85:2149-2154 (1963); Vale *et al.*, *Science* 213, 1394-1397 (1981), and Marke *et al. J. Am Chem. Sci.* 103, 3178 (1981)).

Synthetic methods are also described in "Principles of Peptide Synthesis" M. Bodansky Ed. (Spring-Verlag 1984). These and other methods of peptide synthesis are also exemplified by U.S. Patent Nos. 3,862,925, 3,842,067, 3,972,859, 4,105,602, 4,683,291, 4,244,946 and 4,305,872.

Compounds may also be synthesized using manual or automatic techniques, for example, an Applied BioSystems 430A Peptide Synthesizer (Foster City, California) or a Biosearch SAM 11 automatic peptide synthesizer (Biosearch, Inc., San Rafael, California).

Compounds of the present invention and compositions containing them find use in numerous therapeutic and prophylactic applications in the prevention and treatment of bone reduction related to a disease. Compounds can thus be used as treatments to promote bone growth, in the treatment of osteoporosis, for example, by any suitable route. The preferred routes are suitable for delivery of polypeptide-type compounds to the bloodstream of a subject, bearing in mind proper storage and handling conditions required for polypeptides such as those described herein.

Thus the present invention also provides compositions containing an effective amount of compounds of the present invention, including the nontoxic addition salts, amides and esters thereof, which may, alone, serve to provide the treatment benefits described above. Such compositions can also be provided together with physiologically tolerable liquid, gel or solid diluents, adjuvants and excipients.

In the above examples involving subsequences of about 125 nmol of polypeptide per kg of bodyweight of animal was used per administration. In practice, particularly as human subjects are concerned, the daily dosage may well be between 0.01 and 300 mg or more per kg of bodyweight. More preferably, the dosage would be in the neighborhood of from about 0.1 to about 30 mg per kg of bodyweight. It may be that the preferred frequency of administration would be greater or less than once per day, depending upon the route of administration, convenience, and the variation of effectiveness of treatment with frequency of and amount used per administration. The dosage administered also depends on the subject and to which effect such administration is to give. The dosage of any one or more of the compounds will depend on many factors including the specific compound or combination of compounds being utilized, the mode of administration, and the mammal being treated. Dosages of a particular compound or combination of compounds can be determined using conventional considerations; for example, by customary comparison of the differential activities of the subject compounds and that of a known agent, that is, by means of an appropriate pharmacological protocol in which, for example, bone density of subjects is measured over time.

Pharmaceutical preparations include any of the compounds prepared as an injectable solution, including an injectable solution prepared just prior to use, for promoting bone growth and/or treatment of osteoporosis. An injectable can be either a liquid solution or suspension; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active polypeptide is often mixed with diluents and excipients which are physiologically tolerable and compatible with the polypeptide. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired, the compositions can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH buffering agents, and the like.

Pharmaceutical preparations include the employment of the compounds in admixture with conventional excipients, that is, pharmaceutically acceptable organic or inorganic carrier substances which do not deleteriously react with the compounds, and which possibly enhance the storage and handling stability of the compounds. The preparative procedure may include the sterilization of the pharmaceutical preparations. The compounds may be mixed with auxiliary agents such as lubricants, preservatives, stabilizers, salts for influencing osmotic pressure, etc., which do not react deleteriously with the compounds.

The compositions are conventionally administered parenterally, by injection, for example either subcutaneously or intravenously. Additional formulations which are suitable for other modes of administration include suppositories, intranasal aerosols, and, in some cases, oral formulations. For suppositories, traditional binders and excipients may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%.

Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills capsules, sustained release formulations, or powders, and contain 10% - 95% of active ingredient, preferably 25% - 70%. These oral formulations include formulations designed to protect the peptide until it can be absorbed.

The peptide compounds may be formulated into the compositions as neutral or salt forms. Pharmaceutically acceptable non-toxic salts include the acid addition salts (formed with the free amino groups) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The compounds of the invention can be homopolymerized to themselves (i.e., (peptide)_n) or, heteropolymerized to one another. The compounds can also be conjugated to biocompatible polymeric compounds, such as BIOPOL™ (WR Grace & Co.-Conn.).

If prepared using recombinant techniques, a DNA sequence encoding a desired polypeptide of the present invention is synthesized using standard automated techniques, or the coding sequences or portions thereof are retrieved from cDNA or genomic libraries. This DNA is ligated into suitable expression vectors and these vectors are transformed into appropriate hosts. A variety of expression vector/host cell systems can be used, including both procaryotic and eukaryotic culture systems.

Procaryotes most frequently are represented by various strains of *E. coli*. However, other microbial strains may also be used, such as bacilli, for example *bacillus subtilis*, various species of *Pseudomonas*, or other bacterial strains. In such procaryotic systems, plasmid vectors which contain replication origins, and control sequences derived from a species compatible with the host are used. For example, *E. coli* is typically transformed using derivatives of pBR322, a plasmid derived from an *E. coli* species (Bolivar et al., (1977) *Gene* 2:95. Commonly used procaryotic control sequences, which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta-lactamase (penicillinase), lactose (lac) promoter systems (Chang et al., (1977) *Nature* 198:1056), the tryptophan (trp) promoters system (Goeddel et al., (1990) *Nucleic Acids Res* 8:4057), and the lambda-derived P_L promoter and N-gene ribosome binding site (Shimatake et al., (1981) *Nature* 292:128). However, any available promoter system compatible with prokaryotes can be used.

The expression systems useful in the eukaryotic systems of the invention comprise promoters derived from appropriate eukaryotic genes. A class of promoters useful

in yeast, for example, include promoters for synthesis of glycolytic enzymes, including alcohol dehydrogenase promoters, glyceraldehyde-3-phosphate dehydrogenase promoter (Holland & Holland, (1980) *J Biol Chem* 25:2596), alpha-factor promoter (Bitter et al., (1984) *Proc Natl Acad Sci* 81:5330), the gal promoter (Johnston & David, (1984) *Mol Cell Biol* 4:1440) those for 3-phosphoglycerate kinase (Hitzeman et al., (1980) *J. Biol Chem* 256:1385) or the Leu2 gene obtained from YEp13 (Broach, J., et al., (1978) *Gene* 8:121).

Suitable mammalian promoters include the early and late promoters from SV40 (Fiers et al., (1978) *Nature* 273:113) or other viral promoters such as those derived from polyoma, adenovirus II, bovine papilloma virus or avian sarcoma viruses. Suitable viral and mammalian enhancers are cited above. In the event plant cells are used as an expression system, the nopaline synthesis promoter is appropriate (Depicker, A., et al., (1982) *J Mol Appl Gen* 1:56).

The expression systems are included on replication vectors or are caused to integrate into the chromosome of a recombinant host. For systems wherein the vectors include a replication system, these may be low or high copy number, usually having copy numbers of fewer than about 1000, although in certain situations, runaway vectors may be employed. Whether provided on a vector intended for integration or in a replication system, the sequence encoding a polypeptide of the invention may be ligated in tandem with an amplifying gene such as dihydrofolate reductase, metallothioneins, thymidine kinase, or the like. In procaryotic systems, both the amplifying gene and the target gene can be under the regulation of the same transcriptional and translational regulatory regions.

Usually, the vector will include a marker which allows for selection of host cells containing the expression system; the nature of these markers depends on the host and is understood in the art. In addition to required regulators such as promoters, additional sequences such as enhancers can also be employed to enhance the level of transcription. If the polypeptide is to be secreted, an upstream sequence encoding signal peptides such as those described in U.S. Pat. Nos. 4,336,336; 4,338,397; and 4,546,082 may be employed. The signal sequence is enzymatically cleaved as the polypeptide product is secreted.

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, S.N., (1972) *Proc Natl Acad Sci USA* 69:2110; or the RbCl method described in Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1982) Cold Spring Harbor Press, p. 254 is used for prokaryotes or other cells which contain substantial cell wall barriers. Infection with *Agrobacterium tumefaciens* (Shaw, C.H., (1938) et al., *Gene* 23:315) is used for certain plant cells. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, (1978) *Virology* 52:2:546 is preferred. Transformations into yeast are carried out, for example, according to the method of Van Solingen, P., et al., (1977) *J Bacter* 130:946; and Hsiao, C.L., et al., (1979) *Proc Natl Acad Sci USA* 76:3829.

In general, after construction of a suitable expression system, the system is transfected into the appropriate host and successful transformants are selected by markers contained on the expression vectors. Successfully transformed colonies are then cultured in order to produce the desired polypeptide. It is sometimes preferred that a promoter which

5 can be controlled by regulating conditions in the environment be used so that the cells can be grown under conditions where the gene encoding the desired polypeptide of the invention is not expressed, and then production of the polypeptide induced by appropriate manipulation of conditions. For example, if the trp promoter is used in *E. coli*, the cells are grown in the presence of tryptophan and expression is then induced by diminution of tryptophan

10 concentration or by addition of a tryptophan analogue such as indolylacetic acid. If the gene is under control of the PL promoter, the cells are grown at relatively low temperature, such as at about 35°C., to a suitable cell density, and the temperature is then elevated to activate this promoter. If produced in bacterial hosts as a mature intracellular polypeptide, the N-terminal methionine may or may not be cleaved. In mammalian systems, for example, the use of the

15 metallothionein promoter permits induction by addition of heavy metals or glucocorticoids. This protocol is preferred to prevent premature accumulation of the polypeptide which might be harmful to the growth of the cell.

The polypeptide can be produced intracellularly, or in secreted form by construction of vectors in which the peptide is preceded by a signal peptide workable in the

20 appropriate host.

The polypeptide is recovered from the medium or from the cells using suitable techniques generally known in the art, and purified by, for example, ion exchange chromatography, ammonium sulfate precipitation, gel permeation chromatography, and so forth.

25 It will of course be understood, that antibodies to any of the polypeptides disclosed herein could be generated, as described in connection with the "normal" polypeptide (SEQ ID NO:1). Methodology and products can be developed using an antibody to a polypeptide for use in detecting the polypeptide with which the antibody binds. Methodology and products can be developed using an antibody to a polypeptide for use in

30 detecting the polypeptide with which the antibody binds.

For example, an antibody can be linked to or conjugated with a reporter system which is set up to indicate positively binding of the polypeptide to the antibody. Well known reporter systems include radioimmuno assays (RIAs) or immunoradiometric assays (IRMAs). Alternatively, an enzyme-linked immunosorbent assay (ELISA) would have in

35 common with RIAs and IRMAs a relatively high degree of sensitivity, but would generally not rely upon the use of radioisotopes. A visually detectable substance may be produced or at least one detectable in a spectrophotometer. An assay relying upon fluorescence of a substance bound by the enzyme being assayed could be used. It will be appreciated that there are a number of reporter systems which may be used, according to the present

invention, to detect the presence of a particular polypeptide. With standardized sample collection and treatment, polypeptide presence above a threshold amount in blood serum could well be determined.

- Such an antibody-linked reporter system could be used in a method for
- 5 determining whether blood serum of a subject contains a deficient amount of the polypeptide. Given a normal threshold concentration of such a polypeptide in blood serum of a given type of subject, test kits could thus be developed.

- All references, including issued patents and pending patent applications, described above are incorporated herein by reference. This application also incorporates by
- 10 reference the specifications of United States Patent Application No. 031,386, filed March 12, 1993; No. 120,217 filed September 13, 1993; No. 302,485 filed September 12, 1994; and No. 487,074 filed June 7, 1995.

SECRET

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: GENSCI REGENERATION SCIENCES INC.
(B) STREET: 201 - 5090 Explorer Drive
(C) CITY: Mississauga
(D) PROVINCE: Ontario
(E) COUNTRY: CA
(F) POSTAL CODE (ZIP) : L4W 4T9

(A) NAME: TAM, Cherk Shing
(B) STREET: 1072 Rectory Lane
(C) CITY: Oakville
(D) PROVINCE: Ontario
(E) COUNTRY: CA
(F) POSTAL CODE (ZIP) : L6M 2B7

(ii) TITLE OF INVENTION: BONE STIMULATING FACTOR

(iii) NUMBER OF SEQUENCES: 62

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3 1/2 inch, 1.4 Mb storage
(B) COMPUTER: COMPAQ, IBM PC compatible
(C) OPERATING SYSTEM: MS-DOS 5.1
(D) SOFTWARE: WORD PERFECT

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/763,458
(B) FILING DATE: 11-DEC-1996

(2) INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys
1 5 10 15

Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp
20 25 30

Gln Asn Gln Pro
35

(2) INFORMATION FOR SEQ ID NO:2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

662090-466666

(A) NAME/KEY: Modified site

(D) OTHER INFORMATION: /note= "Xaa is N-acetyl glycine"

Xaa Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys
1 5 10 15

Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp
20 25 30

(2) INFORMATION FOR SEQ ID NO:3

(A) LENGTH: 36 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Ala Lys Ile Lys
1 5 10 15

Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp
20 25 30

(2) INFORMATION FOR SEQ ID NO:4

(A) LENGTH: 30 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys
1 5 10 15

Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val
20 25 30

(2) INFORMATION FOR SEQ ID NO:5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys
1 5 10 15

Pro Asn Thr Leu His Lys Lys Ala Ala
20 25

(2) INFORMATION FOR SEQ ID NO:6

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys
1 5 10 15

Pro Asn Thr Leu
20

(2) INFORMATION FOR SEQ ID NO:7

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:8

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8

Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:9

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9

Arg Thr Asn Glu His Thr Ala Asp Cys Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:10

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10

Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn Gln
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:11

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11

Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:12

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12

Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln
1 5 10

(2) INFORMATION FOR SEQ ID NO:13

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13

Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp
1 5 10

(2) INFORMATION FOR SEQ ID NO:14

- (i) SEQUENCE CHARACTERISTICS:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14

(2) INFORMATION FOR SEO ID NO:15

(xi) SEQUENCE DESCRIPTION: SEO ID NO:15

(2) INFORMATION FOR SEO ID NO:16

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16

(2) INFORMATION FOR SEQ ID NO:17

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17

GGG	ATC	GGA	AAA	CGA	ACA	AAT	GAA	CAT	ACG	GCA	GAT	TGT	AAA	ATT	AAA	48
Gly	Ile	Gly	Lys	Arg	Thr	Asn	Glu	His	Thr	Ala	Asp	Cys	Lys	Ile	Lys	
1				5					10					15		
CCG	AAC	ACC	TTG	CAT	AAA	AAA	GCT	GCA	GAG	ACT	TTA	ATG	GTC			90
Pro	Asn	Thr	Leu	His	Lys	Lys	Ala	Ala	Glu	Thr	Leu	Met	Val			

20

25

30

(2) INFORMATION FOR SEQ ID NO:18

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 75 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18

GGG ATC GGA AAA CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA ATT AAA 48
 Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys
 1 5 10 15

CCG AAC ACC TTG CAT AAA AAA GCT GCA 75
 Pro Asn Thr Leu His Lys Lys Ala Ala
 20 25

(2) INFORMATION FOR SEQ ID NO:19

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 60 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19

GGG ATC GGA AAA CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA ATT AAA 48
 Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys
 1 5 10 15

CCG AAC ACC TTG 60
 Pro Asn Thr Leu
 20

(2) INFORMATION FOR SEQ ID NO:20

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20

GGG ATC GGA AAA CGA ACA AAT GAA CAT ACG GCA GAT TGT AAA ATT 45
 Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile
 1 5 10 15

(2) INFORMATION FOR SEQ ID NO:21

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 42 base pairs

662290 4382360

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21

(2) INFORMATION FOR SEQ ID NO:22
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22

(2) INFORMATION FOR SEQ ID NO:23
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 108 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23

CCG AAC ACC TTG CAT AAA AAA GCT GCA GAG ACT TTA ATG GTC CTT GAC 96
Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp
20 25 30

(2) INFORMATION FOR SEQ ID NO:24
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

```
(ix) FEATURE:
      (A) NAME/KEY: Modified site
      (B) LOCATION: ...2
```

(D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

(A) NAME/KEY: Modified site

(B) LOCATION: 9...

(D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24

Xaa	Thr	Asn	Glu	His	Thr	Ala	Asp	Cys	Xaa
1				5					10

(2) INFORMATION FOR SEQ ID NO:25

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified site

(B) LOCATION: ...2

(D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

(A) NAME/KEY: Modified site

(B) LOCATION: 9...

(D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25

Xaa	Thr	Asn	Glu	His	Thr	Ala	Glu	Cys	Xaa
1				5					10

(2) INFORMATION FOR SEQ ID NO:26

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified site

(B) LOCATION: ...2

(D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

(A) NAME/KEY: Modified site

(B) LOCATION: 9...

(D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26

Xaa	Thr	Gln	Glu	His	Thr	Ala	Glu	Cys	Xaa
1				5					10

Arg Thr Gln Glu His Thr Ala Asp Cys Lys
1 5 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31

CGA	ACA	AAT	GAA	CAT	ACG	GCA	GAA	TGT	AAA	30
Arg	Thr	Asn	Glu	His	Thr	Ala	Glu	Cys	Lys	
1				5				10		

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32

CGA	ACA	CAA	GAA	CAT	ACG	GCA	GAA	TGT	AAA	30
Arg	Thr	Gln	Glu	His	Thr	Ala	Glu	Cys	Lys	
1				5					10	

(2) INFORMATION FOR SEQ ID NO:33
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33

CGA ACA CAA GAA CAT ACG GCA GAT TGT AAA 30
Arg Thr Gln Glu His Thr Ala Asp Cys Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:34
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

```
(ix) FEATURE:
      (A) NAME/KEY: Modified site
      (B) LOCATION: ...2
      (D) OTHER INFORMATION: /note= "Xaa is N-acetyl alanine"
```

- (ix) FEATURE:
 (A) NAME/KEY: Modified site
 (B) LOCATION: 9...
 (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34

Xaa Thr Asn Glu His Thr Ala Asp Cys Xaa
 1 5 10

(2) INFORMATION FOR SEQ ID NO:35

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

- (ix) FEATURE:
 (A) NAME/KEY: Modified site
 (B) LOCATION: ...2
 (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

- (ix) FEATURE:
 (A) NAME/KEY: Modified site
 (B) LOCATION: 9...
 (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35

Xaa Thr Gln Ala His Thr Ala Asp Cys Xaa
 1 5 10

(2) INFORMATION FOR SEQ ID NO:36

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

- (ix) FEATURE:
 (A) NAME/KEY: Modified site
 (B) LOCATION: ...2
 (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

- (ix) FEATURE:
 (A) NAME/KEY: Modified site
 (B) LOCATION: 9...
 (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36

Xaa Thr Asn Glu Ala Thr Ala Asp Cys Xaa
 1 5 10

(2) INFORMATION FOR SEQ ID NO:37

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: ...2
- (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 9...
- (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37

Xaa Thr Asn Glu His Thr Ala Ala Cys Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:38

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: ...2
- (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 9...
- (D) OTHER INFORMATION: /note= "Xaa is alaninamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38

Xaa Thr Asn Glu His Thr Ala Asp Cys Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:39

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: ...2
- (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 9...
- (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39

Xaa Ala Asn Glu His Thr Ala Asp Cys Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: ...2
- (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 9...
- (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40

Xaa Thr Ala Glu His Thr Ala Asp Cys Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:41

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: ...2
- (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 9...
- (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41

Xaa Thr Asn Glu His Ala Ala Asp Cys Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:42

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

```
(ix) FEATURE:
      (A) NAME/KEY: Modified site
      (B) LOCATION: 9...
      (D) OTHER INFORMATION: /note= "Xaa is lysinamide"
```

Xaa Thr Asn Glu His Thr Gly Asp Cys Xaa
1 5 10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

```
(ix) FEATURE:
(A) NAME/KEY: Modified site
(B) LOCATION: 9...
(D) OTHER INFORMATION: /note= "Xaa is lysinamide"
```

Xaa Thr Asn Glu His Thr Ala Asp Tyr Xaa
1 5 10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

```
(ix) FEATURE:
      (A) NAME/KEY: Modified site
      (B) LOCATION: 9...
      (D) OTHER INFORMATION: /note= "Xaa is lysinamide"
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44

Xaa Thr Gln Glu His Thr Ala Glu Ala Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:45

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: ...2
- (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 9...
- (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45

Xaa Thr Gln Glu His Thr Ala Glu Tyr Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:46

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: ...2
- (D) OTHER INFORMATION: /note= "Xaa is N-acetyl arginine"

(ix) FEATURE:

- (A) NAME/KEY: Modified site
- (B) LOCATION: 9...
- (D) OTHER INFORMATION: /note= "Xaa is lysinamide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46

Xaa Thr Gln Glu His Thr Ala Glu Ser Xaa
1 5 10

(2) INFORMATION FOR SEQ ID NO:47

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47

6622904362260

Arg Ala Asn Glu His Thr Ala Asp Cys Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:48
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48

Arg Thr Ala Glu His Thr Ala Asp Cys Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:49
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49

Arg Thr Asn Glu His Ala Ala Asp Cys Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:50
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50

Arg Thr Asn Glu His Thr Gly Asp Cys Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:51
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51

Arg Thr Asn Glu His Thr Ala Asp Tyr Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:52
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids

662030 4532250

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52

(2) INFORMATION FOR SEQ ID NO:53
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

Arg Thr Gln Glu His Thr Ala Glu Tyr Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:54
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

Arg Thr Gln Glu His Thr Ala Glu Ser Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:55
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

CGA	GCA	AAT	GAA	CAT	ACG	GCA	GAT	TGT	AAA
Arg	Ala	Asn	Glu	His	Thr	Ala	Asp	Cys	Lys
1				5					10

(2) INFORMATION FOR SEQ ID NO:56
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56

CGA ACA GCA GAA CAT ACG GCA GAT TGT AAA 30
Arg Thr Ala Glu His Thr Ala Asp Cys Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:57
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57

CGA ACA AAT GAA CAT GCA GCA GAT TGT AAA 30
Arg Thr Asn Glu His Ala Ala Asp Cys Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:58
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58

CGA ACA AAT GAA CAT ACA GGG GAT TGT AAA 30
Arg Thr Asn Glu His Thr Gly Asp Cys Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:59
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59

CGA ACA AAT GAA CAT ACA GCA GAT TAT AAA 30
Arg Thr Asn Glu His Thr Ala Asp Tyr Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:60
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60

662990495260

CGA ACA CAA GAA CAT ACA GCA GAA GCA AAA 30
Arg Thr Gln Glu His Thr Ala Glu Ala Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:61
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61

CGA ACA CAA GAA CAT ACA GCA GAA TAT AAA 30
Arg Thr Gln Glu His Thr Ala Glu Tyr Lys
1 5 10

- (2) INFORMATION FOR SEQ ID NO:62
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62

CGA ACA CAA GAA CAT ACA GCA GAA TCT AAA 30
Arg Thr Gln Glu His Thr Ala Glu Ser Lys
1 5 10

CGA ACA CAA GAA CAT ACA GCA GAA TCT AAA

CLAIMS

1. A compound having bone stimulatory activity in mammals, the compound being derived from a polypeptide having the charge pattern of the amino acid side chain charges provided by the amino acid sequence corresponding to SEQ ID NO:9 wherein the amino acid sequence can have up to 35 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1.
2. The compound of claim 1 wherein the compound includes a backbone which is substantially isosteric with that provided by the peptide backbone of the amino acid sequence corresponding to SEQ ID NO:9, wherein the amino acid sequence can have up to 30, 25, 20, 15, or 10 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1, and wherein the compound can be a polypeptide.
3. The compound of claim 1 wherein the charge pattern of the compound consists essentially of that provided by the amino acid sequence corresponding to SEQ ID NO:9.
4. The compound of claim 3 having the charge pattern and spacing of that provided by the amino acid sequence corresponding to SEQ ID NO:9.
5. The compound of claim 1, including substitutions of the sequence corresponding to SEQ ID NO:9 which retain bone stimulatory activity in mammals.
6. The compound of claim 5, wherein the compound is a polypeptide and a said sequence from which the polypeptide is derived consists of up to 25 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1 and conjugates of one or more selected sequences; and wherein a said sequence from which the polypeptide is derived can consist of up to 20 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1 and conjugates of one or more selected sequences; and wherein a said sequence from which the polypeptide is derived can consist of up to 15 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1 and conjugates of one or more selected sequences; and wherein a said sequence from which the polypeptide is derived can consist of up to 10 consecutive amino acids selected from the sequence corresponding to SEQ ID NO:1 and conjugates of one or more selected sequences.
7. The compound of claim 3, wherein the compound is a polypeptide having an amino acid sequence selected from that corresponding to SEQ ID NO:9; SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:39; SEQ ID NO:40; SEQ ID NO:41; SEQ ID NO:42; SEQ ID NO:43; SEQ ID NO:44; SEQ ID NO:45 and SEQ ID NO:46, including substitutions which retain bone stimulatory activity in mammals.
8. A compound having bone stimulatory activity in mammals, the compound having the charge distribution of the side chain charges provided by the amino acid sequence corresponding to SEQ ID NO:9 and having up to about 83% sequence homology with SEQ ID NO:1.
9. The compound of claim 8, wherein the compound is a polypeptide up to 30 amino acids in length, and conjugates thereof, wherein the compound can have up to about 69% sequence homology with SEQ ID NO:1; or wherein the compound is a polypeptide up to 25 amino acids in

length, and conjugates thereof, wherein the compound can have up to about 55% sequence homology with SEQ ID NO:1; or wherein the compound is a polypeptide up to about 20 amino acids in length, and conjugates thereof, and wherein the compound can have up to about 42% sequence homology with SEQ ID NO:1; or wherein the compound is a polypeptide up to about 15 amino acids in length, and conjugates thereof, and wherein the compound can have up to about 28% sequence homology with SEQ ID NO:1; or wherein the compound is a polypeptide up to about 10 amino acids in length, and conjugates thereof.

10. A polypeptide having bone stimulatory activity in mammals, the polypeptide having the charge distribution of the side chain charges provided by the amino acid sequence identified as SEQ ID NO:9 and including at least one non-conservative substitution at position number 2, 3, 6 or 9; wherein the polypeptide can have an amino acid sequence derived from SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:42, or SEQ ID NO:43, and substitutions thereof which retain bone stimulatory activity in mammals.

11. A polypeptide comprising an amino acid sequence corresponding to SEQ ID NO:1 or SEQ ID NO:3 with (a) from one to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:1 or SEQ ID NO:3, (b) one to about 22 amino acids deleted from the C-terminus of SEQ ID NO:1 or SEQ ID NO:3, or both (a) and (b); or a functionally equivalent homologue which promotes bone growth in mammals; wherein the polypeptide can comprise an amino acid sequence corresponding to SEQ ID NO:4 with (a) up to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:4, (b) up to about 16 amino acids deleted from the C-terminus of SEQ ID NO:4, or both (a) and (b); or a functionally equivalent homologue which promotes bone growth in mammals; and wherein the polypeptide can comprise an amino acid sequence corresponding to SEQ ID NO:5 with (a) up to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:5, (b) up to about 11 amino acids deleted from the C-terminus of SEQ ID NO:5, or both (a) and (b); or a functionally equivalent homologue which promotes bone growth in mammals; and wherein the polypeptide can comprise an amino acid sequence corresponding to SEQ ID NO:6 with (a) up to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:6, (b) up to about 5 amino acids deleted from the C-terminus of SEQ ID NO:6, or both (a) and (b); or a functionally equivalent homologue which promotes bone growth in mammals; and wherein the polypeptide can comprise an amino acid sequence corresponding to SEQ ID NO:7 with (a) up to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:7, (b) up to about 1 amino acids deleted from the C-terminus of SEQ ID NO:4, or both (a) and (b); or a functionally equivalent homologue which promotes bone growth in mammals; and wherein the polypeptide can comprise an amino acid sequence corresponding to SEQ ID NO:8 with up to about four 4 amino acids deleted from the N-terminus or a functionally equivalent homologue which promotes bone growth in mammals

12. A polypeptide up to about 20 amino acids in length comprising an amino acid sequence corresponding to SEQ ID NO:9, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53,

or SEQ ID NO:54, or a functionally equivalent homologue thereof which promotes bone growth in mammals.

13. A polypeptide of claim 12, having a charge pattern of the amino acid side chain charges provided by the amino acid sequence corresponding to SEQ ID NO:9.

14. A polypeptide of claim 13 comprising an amino acid sequence corresponding to SEQ ID NO:29 or SEQ ID NO:54.

15. A polypeptide of claim 14 consisting essentially of an amino acid sequence corresponding to SEQ ID NO:29 or SEQ ID NO:54, including protected derivatives thereof.

16. A polypeptide of claim 12, wherein the polypeptide is synthetic and the amino acid sequence has a molecular weight limited to the range of from about 1000 to 4000, or of from about 1000 to 3000, or from about 1000 to 2000, or from about 1000 to about 1500.

17. A first polypeptide having a sequence up to about 30 amino acids in length which first polypeptide is sufficiently duplicative of a second polypeptide comprising an amino acid sequence corresponding to SEQ ID NO:1 or SEQ ID NO:3 with (a) from one to about four 4 amino acids deleted from the N-terminus of SEQ ID NO:1 or SEQ ID NO:3, (b) one to about 22 amino acids deleted from the C-terminus of SEQ ID NO:1 or SEQ ID NO:3, or both (a) and (b), or a functionally equivalent homologue thereof, such that the first polypeptide is encoded by a DNA that hybridizes under stringent conditions with DNA encoding the second polypeptide.

18. A polypeptide which is a conservatively substituted variant of a polypeptide of claim 10.

19. A synthetic polypeptide having in vivo bone stimulatory activity in mammals and which increases mineral content in bones of mammals, having an amino acid sequence which is at least about 19% conserved in relation to the amino acid sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom, or a functionally equivalent homologue; or a said synthetic polypeptide having an amino acid sequence which is at least about 22% conserved in relation to the amino acid sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom; or a said synthetic polypeptide which is at least about 25% conserved in relation to the amino acid sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom; or a said synthetic polypeptide having an amino acid sequence which is at least about 28% conserved in relation to the amino acid sequence identified as SEQ ID NO:1 and having at least one amino acid deleted therefrom.

20. A polypeptide of claim 19 having at least six amino acids deleted from said sequence, or having at least sixteen amino acids deleted from said sequence, or having at least twenty-one amino acids deleted from said sequence, or having at least twenty-six amino acids deleted from said sequence.

21. A polypeptide of claim 20 wherein the polypeptide has a molecular weight in the range of from about 1000 to 4000, or from about 1000 to 3000, or from about 1000 to 2000, or from about 1000 to 1500.

22. A first polypeptide comprising a sequence of amino acids sufficiently duplicative of a second polypeptide comprising an amino acid sequence of claim 19 such that the first polypeptide is

encoded by a DNA that hybridizes under stringent conditions with DNA encoding the second polypeptide.

23. A chimeric bone stimulating factor comprising a polypeptide of claim 6.

24. A method of increasing bone growth in a mammal by administering a therapeutically effective amount of a polypeptide of claim 6.

25. A method of increasing bone growth in a mammal by administering a therapeutically effective amount of a compound of claim 1.

26. The use of a polypeptide of claim 6, for the treatment of osteoporosis.

27. The use of a compound of claim 1, for the treatment of osteoporosis.

28. The use of a polypeptide of claim 6, to promote bone growth in a mammal.

29. The use of a compound of claim 1, to promote bone growth in a mammal.

30. A diagnostic kit for determining the presence of a polypeptide of claim 6, comprising an antibody to a said polypeptide linked to a reporter system wherein the reporter system produces a detectable response when a predetermined amount of the polypeptide and the antibody are bound together.

31. A diagnostic kit for determining the presence of a compound of claim 1, comprising an antibody to a said compound linked to a reporter system wherein the reporter system produces a detectable response when a predetermined amount of the compound and the antibody are bound together.

32. An isolated DNA fragment which encodes the expression of any of the polypeptides of claim 6, and DNA which differs from the fragment due to the degeneracy of the genetic code.

33. A vector comprising a DNA sequence which encodes the expression of any of the polypeptides of claim 6.

34. A process for producing a polypeptide of claim 6, which comprises:

- a) preparing a DNA fragment containing a nucleotide sequence which encodes said polypeptide;
- b) incorporating said DNA fragment into an expression vector to obtain a recombinant DNA fragment which contains said DNA fragment and is capable of undergoing replication;
- c) transforming a host cell with said recombinant DNA fragment to isolate a transformant which can express said polypeptide; and
- d) culturing said transformant to allow the transformant to produce said polypeptide and recovering said polypeptide from resulting cultured mixture.

35. A polypeptide having bone stimulatory activity, the polypeptide comprising an amino acid sequence that has $10 + q$ amino acids, wherein, under physiological conditions, residues numbered n , $n + 4$, $n + 9$ are positively charged amino acids, residues numbered $n + 3$, $n + 7$ are negatively charged amino acids, wherein the remaining amino acids are nonpolar amino acids or uncharged polar amino acids, wherein n is an integer from 1 to $1 + q$ and q is an integer from 0 to 15; and, wherein the polypeptide includes no more than 25 consecutive amino acids corresponding to the amino acid sequence identified as SEQ ID NO:1.

36. The polypeptide of claim 35, wherein $q = 10, 5$ or 0 .
37. The polypeptide of claim 35 wherein each of the remaining amino acids is selected from the group consisting of glycine, alanine, valine, isoleucine, serine, threonine, methionine, asparagine and glutamine.
38. The polypeptide of claim 35, wherein the residue numbered $n + 8$ is cysteine, serine, tyrosine or alanine.
39. The polypeptide of claim 35, wherein the residue numbered n is arginine.
40. The polypeptide of claim 35, wherein the residue numbered $n + 1$ is alanine or threonine.
41. The polypeptide of claim 35, wherein the residue numbered $n + 2$ is alanine, asparagine, or glutamine.
42. The polypeptide of claim 35, wherein the residue numbered $n + 3$ is glutamic acid.
44. The polypeptide of claim 35, wherein the residue numbered $n + 4$ is histidine.
45. The polypeptide of claim 35, wherein the residue numbered $n + 5$ is threonine or alanine.
46. The polypeptide of claim 35, wherein the residue numbered $n + 6$ is glycine or alanine.
47. The polypeptide of claim 35, wherein the residue numbered $n + 7$ is glutamic acid or asparatic acid.
48. The polypeptide of claim 35, wherein the residue numbered $n + 9$ is lysine.
49. The polypeptide of claim 38, wherein the residue numbered $n + 8$ is serine.

ABSTRACT OF THE DISCLOSURE

Polypeptides which stimulate bone growth: Gly Ile Gly Lys Arg Thr Asn Glu His Thr Ala Asp Cys Lys Ile Lys Pro Asn Thr Leu His Lys Lys Ala Ala Glu Thr Leu Met Val Leu Asp Gln Asn Gln Pro and subsequences, particularly Arg Thr Asn Glu His Thr Ala Asp Cys Lys and associated nucleotide sequences, methods of preparation and use.

66000-46660

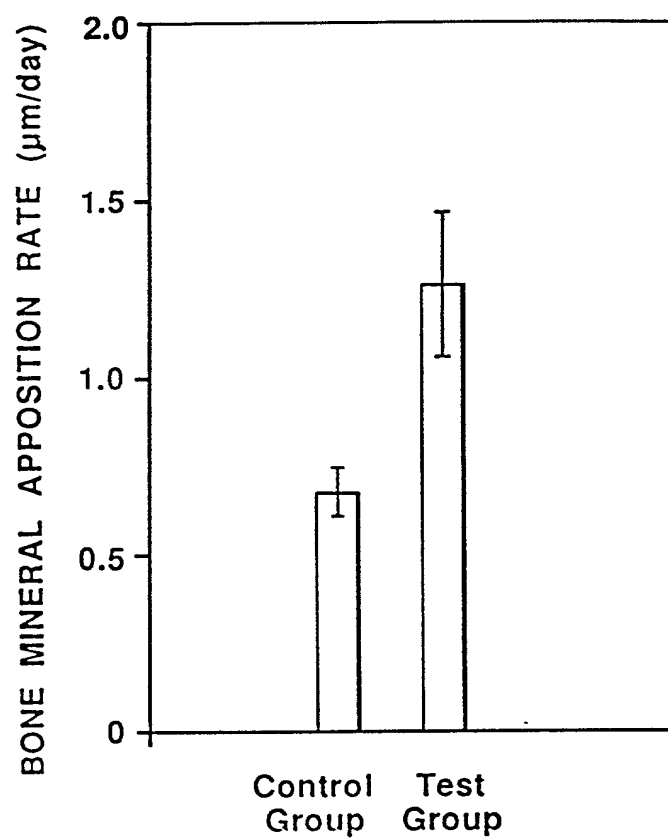


FIG. 1

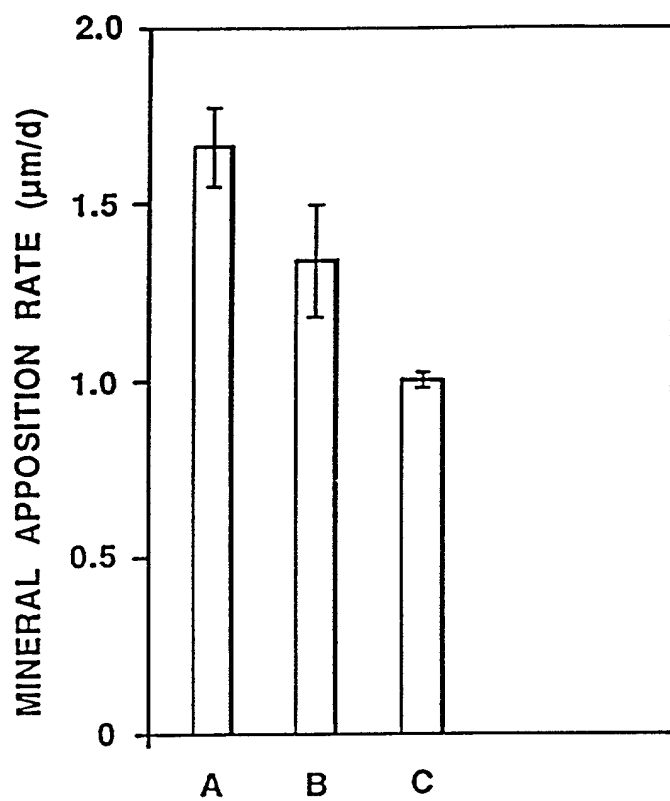


FIG. 7

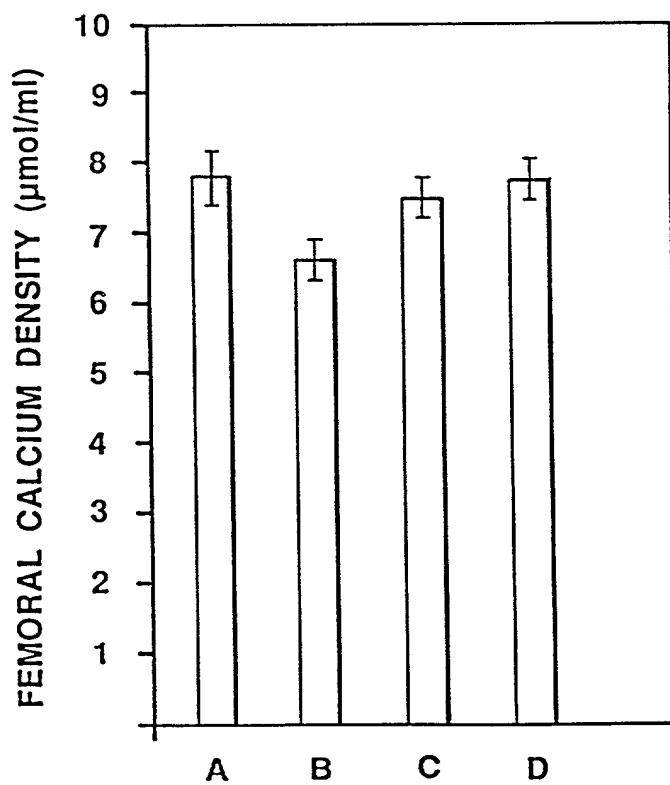


FIG 2

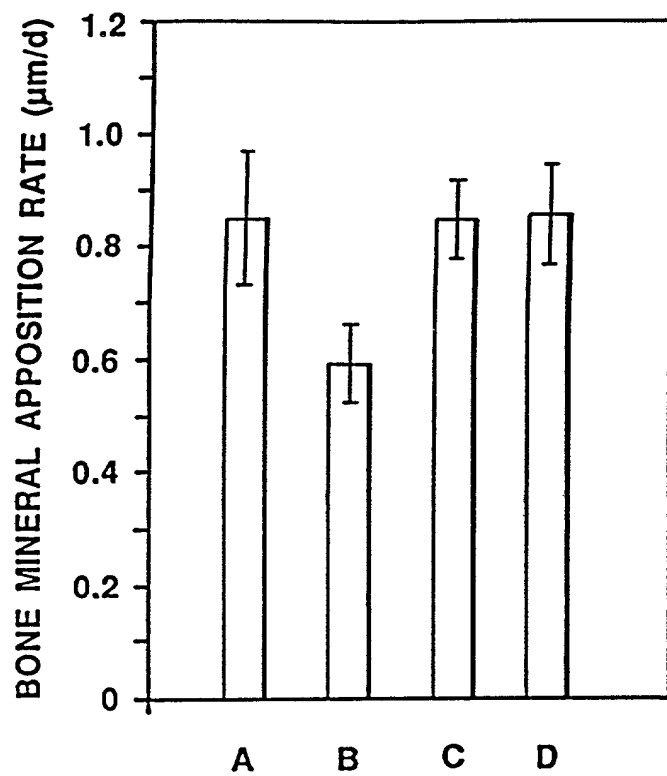


FIG 3

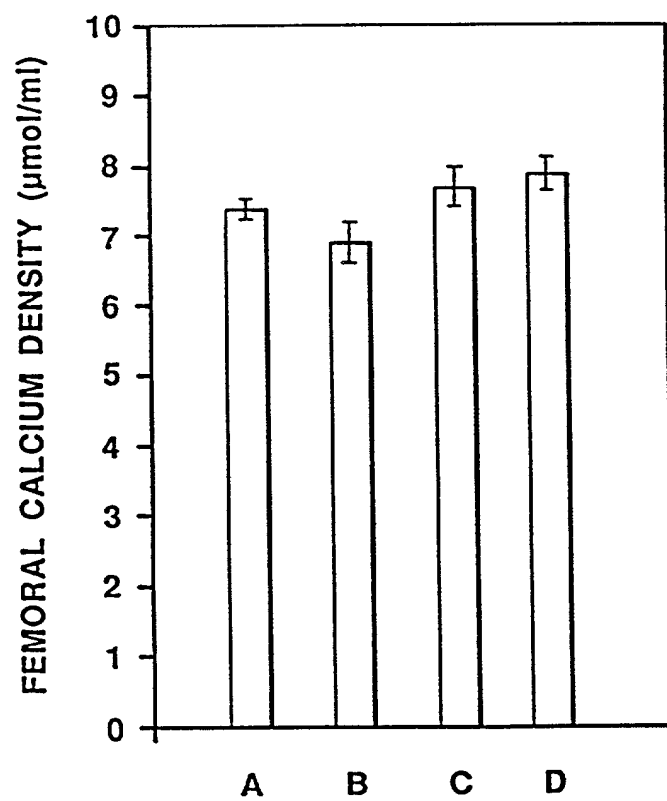


FIG. 4

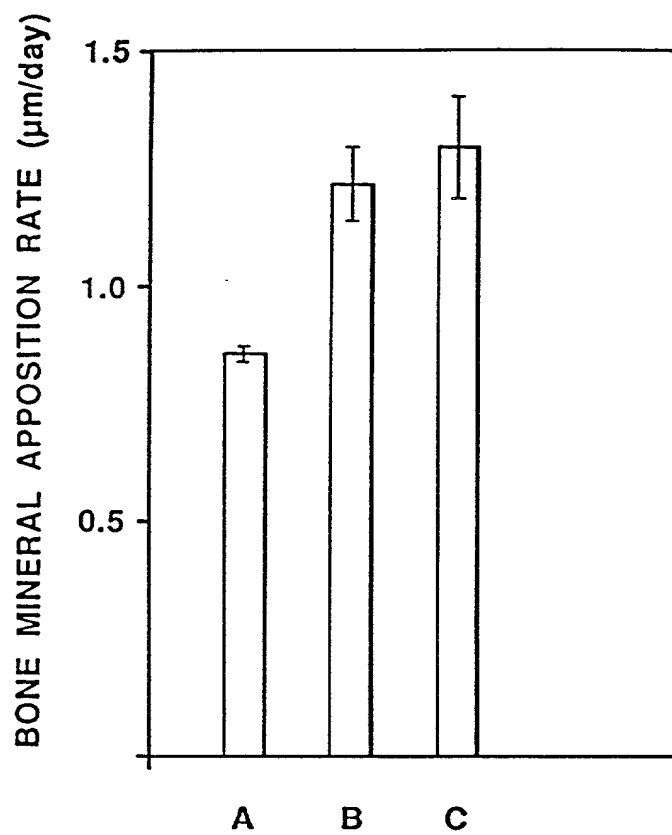


FIG. 5

14K

6.0K

3.5K

2.5K

MARKERS

NORMAL POLYPEPTIDE

MODIFIED POLYPEPTIDE

FIG. 6

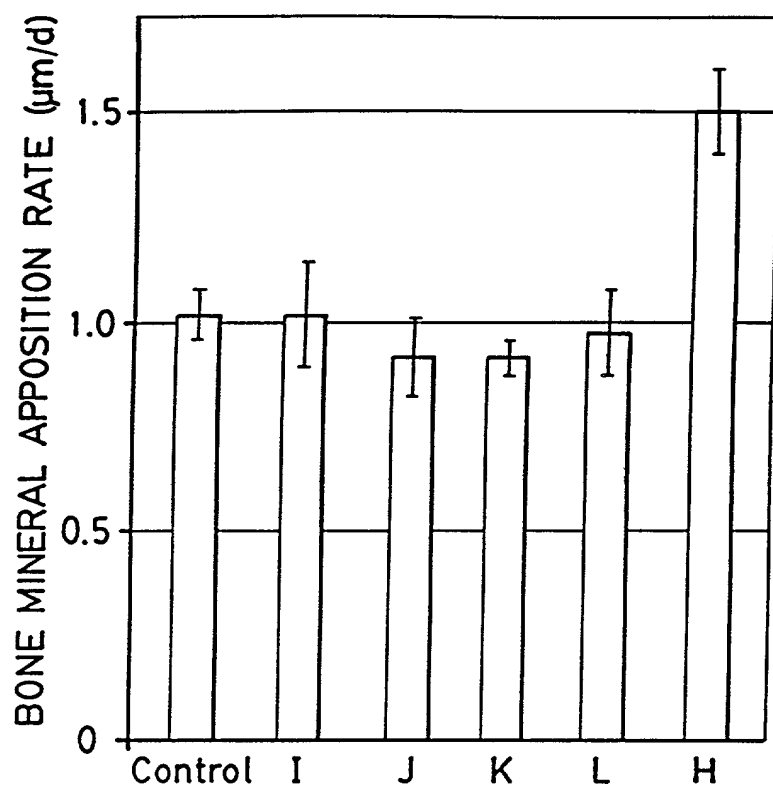


FIG. 12

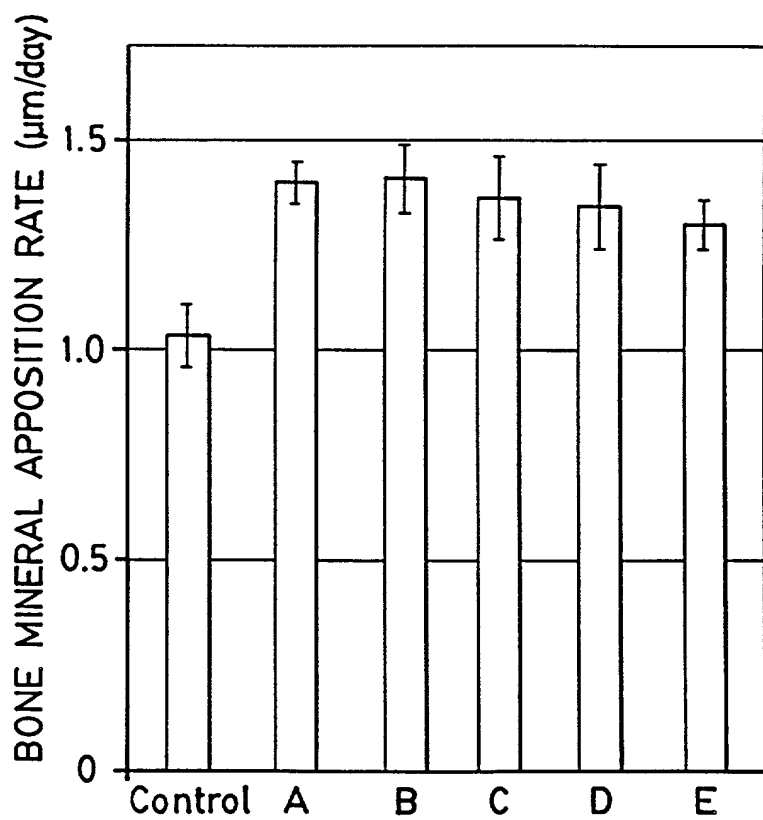


FIG. 8

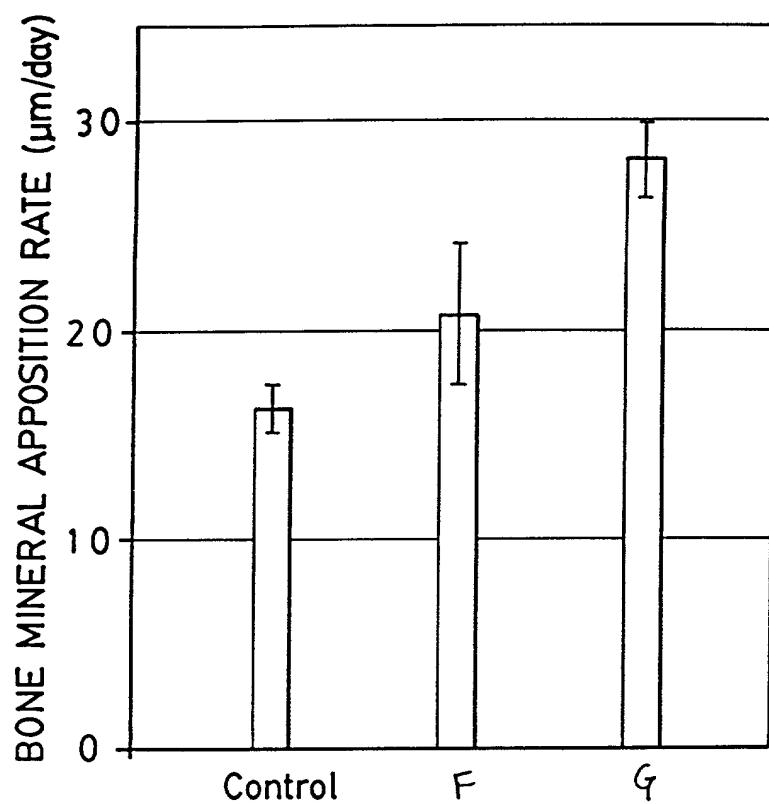


FIG. 9

FIG. 10

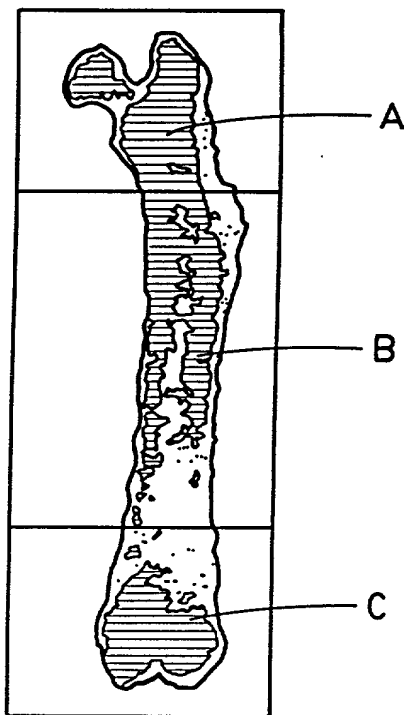
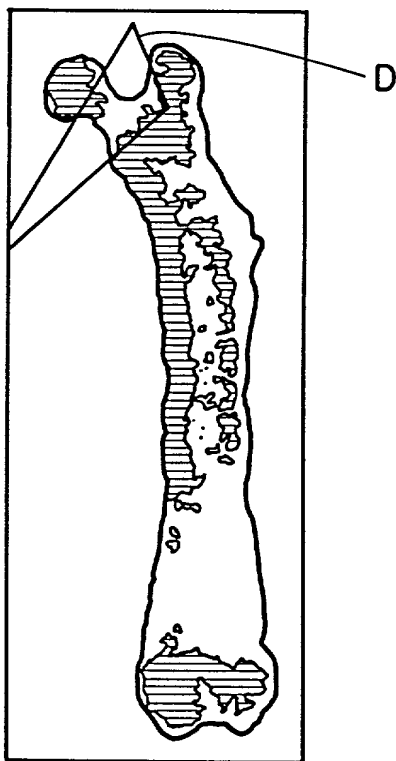


FIG. 11



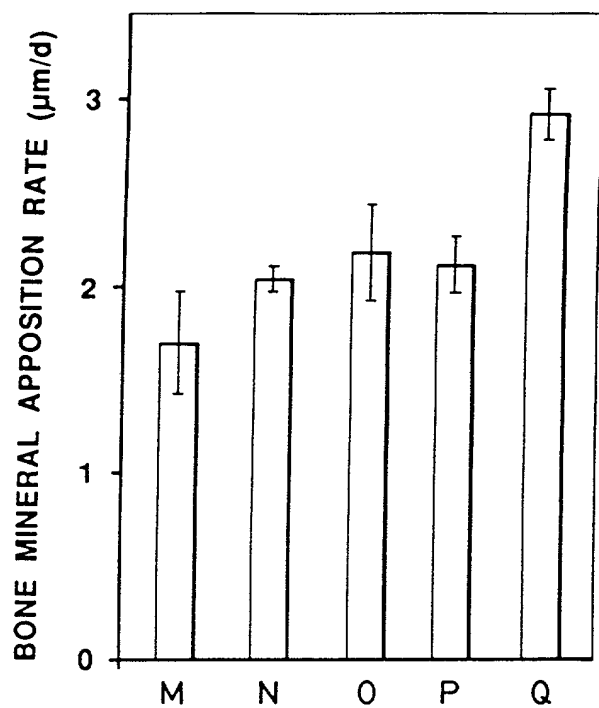


FIG. 13

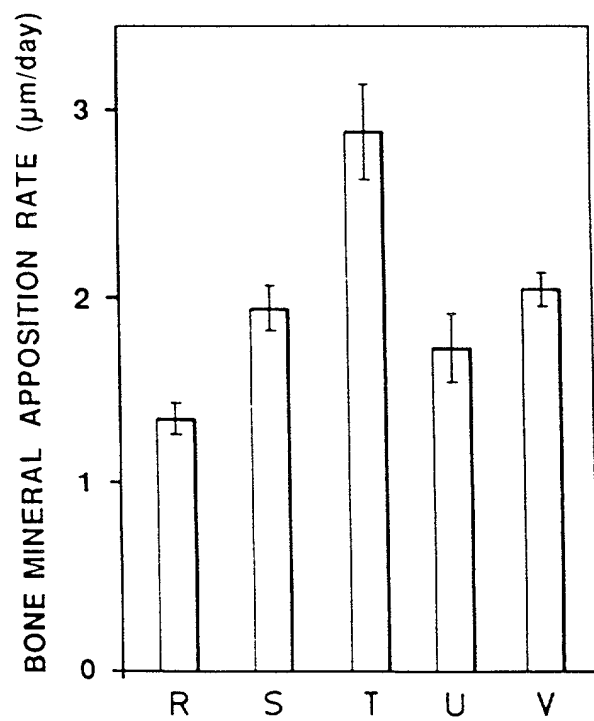


FIG. 14

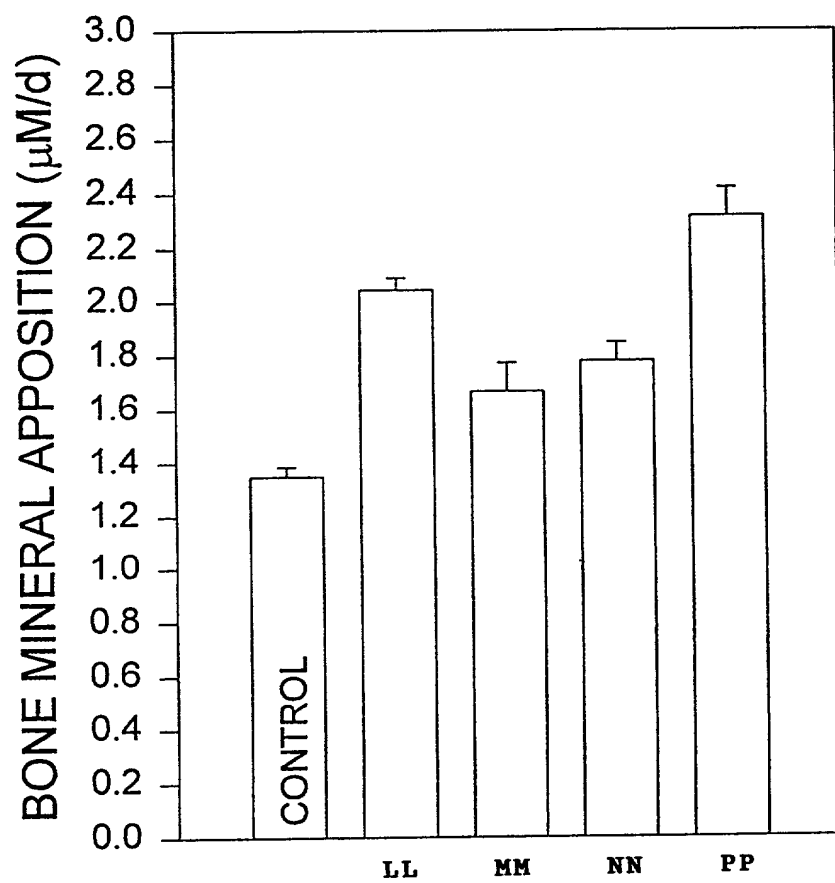


FIG. 15

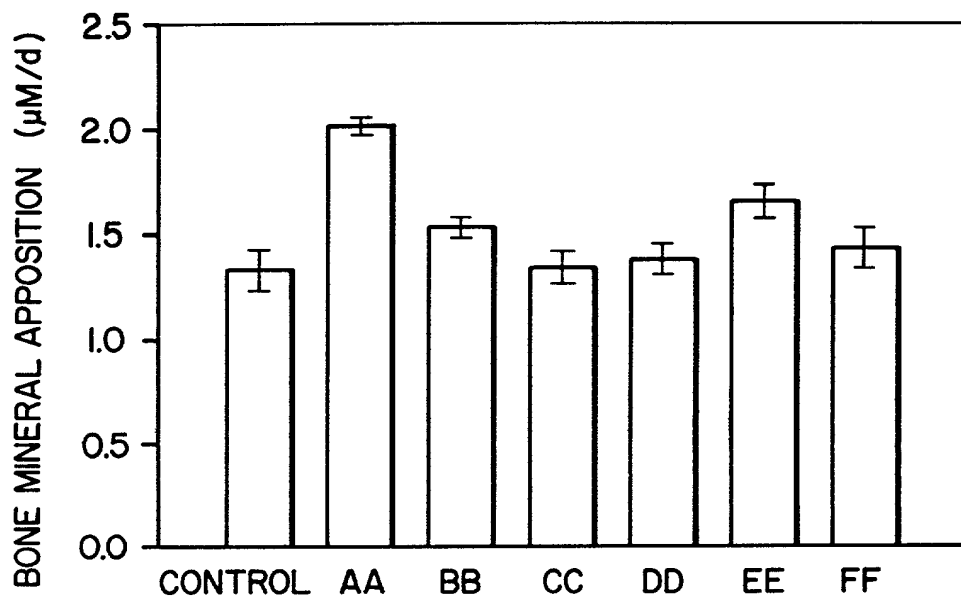


FIG. 16

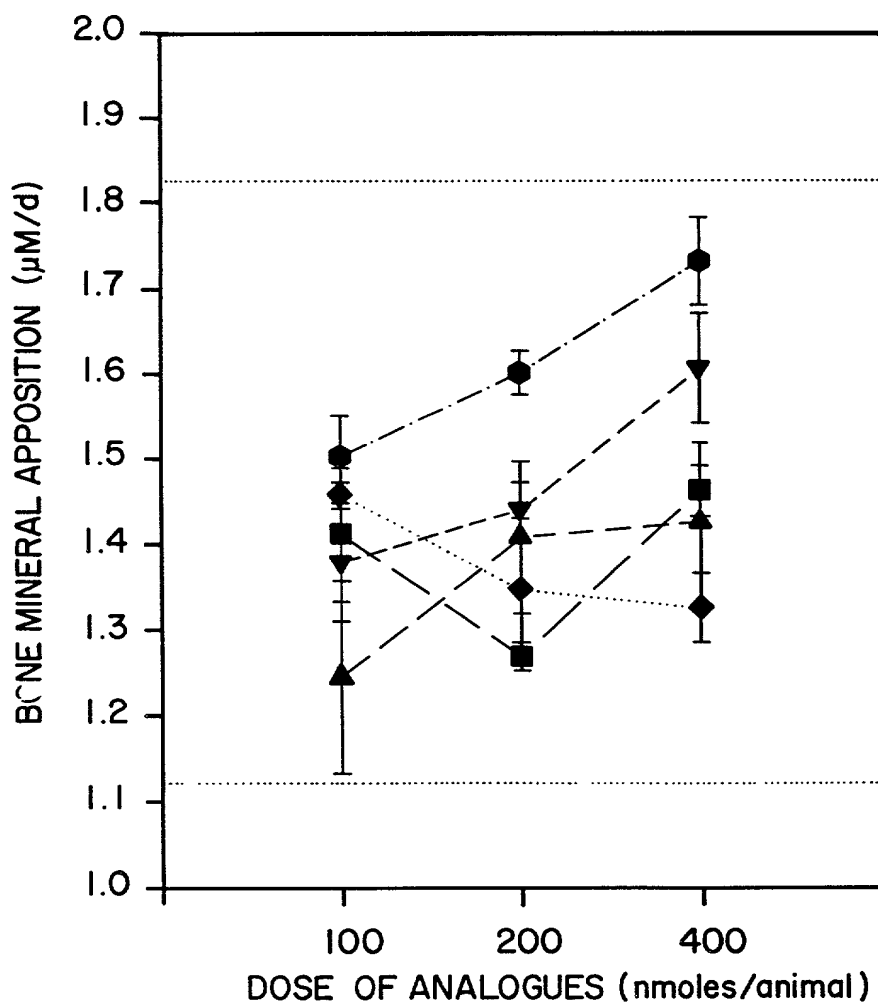


FIG. 17

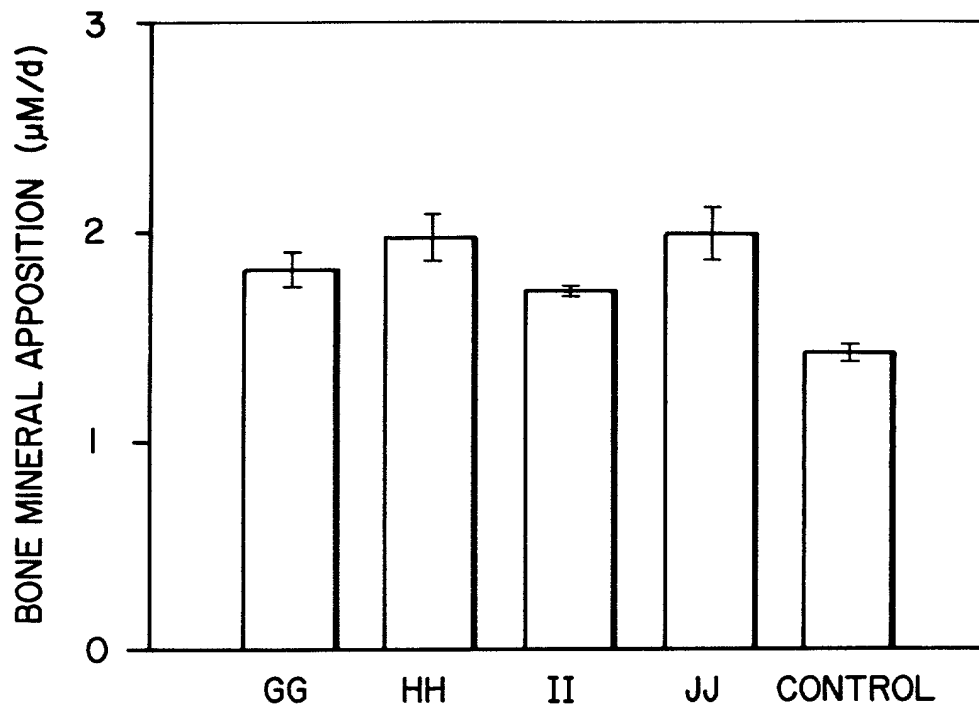


FIG. 18

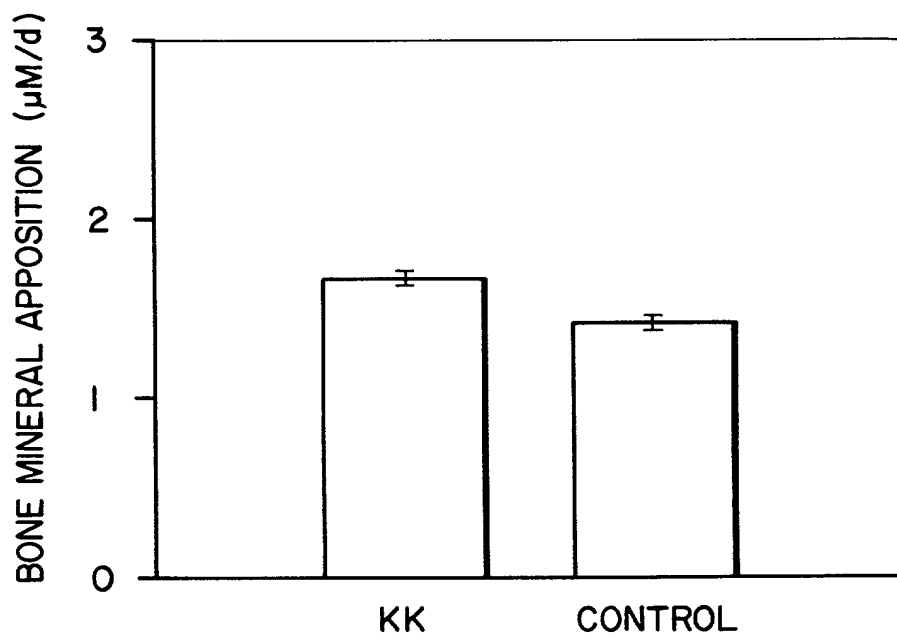


FIG. 19

Genetic

Active Sequences

SEQ ID NO:

1	G	I	G	K	R	5	T	N	E	H	10	A	D	C	K	I	K	P	N	T	L	H	K	K	A	A	E	T	L	M	V	L	D	Q	N	Q	P
	1														15						20					25				30				35			
3	G	I	G	K	R		T	N	E	H	T	A	D	<u>A</u>	K	I	K	P	N	T	L	H	K	K	A	A	E	T	L	M	V	L	D	Q	N	Q	P
4	G	I	G	K	R		T	N	E	H	T	A	D	C	K	I	K	P	N	T	L	H	K	K	A	A	E	T	L	M	V	L	D	Q	N	Q	P
5	G	I	G	K	R		T	N	E	H	T	A	D	C	K	I	K	P	N	T	L	H	K	K	A	A	E	T	L	M	V	L	D	Q	N	Q	P
6	G	I	G	K	R		T	N	E	H	T	A	D	C	K	I	K	P	N	T	L	H	K	K	A	A	E	T	L	M	V	L	D	Q	N	Q	P
7	G	I	G	K	R		T	N	E	H	T	A	D	C	K	I	K	P	N	T	L	H	K	K	A	A	E	T	L	M	V	L	D	Q	N	Q	P
8	G	I	G	K	R		T	N	E	H	T	A	D	C	K	I	K	P	N	T	L	H	K	K	A	A	E	T	L	M	V	L	D	Q	N	Q	P
9					R		T	N	E	H	T	A	D	C	K																						
24			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
25			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
26			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
27			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
39			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
40			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
41			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
42			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
43			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
44			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
45			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				
46			Ac	-	R		T	N	E	H	T	A	D	C	K	-	NH ₂																				

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13																																					
14																																					
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16																																					
34	Ac	-	<u>A</u>				T	N	E	H	T	A	D	C	K	-	NH ₂																				
35	Ac	-	R				T	N	E	H	T	A	D	C	K	-	NH ₂																				
36	Ac	-	R				T	N	E	H	T	A	D	C	K	-	NH ₂																				
37	Ac	-	R				T	N	E	H	T	A	D	C	K	-	NH ₂																				
38	Ac	-	R				T	N	E	H	T	A	D	C	<u>A</u>	-	NH ₂																				

FIGURE 20